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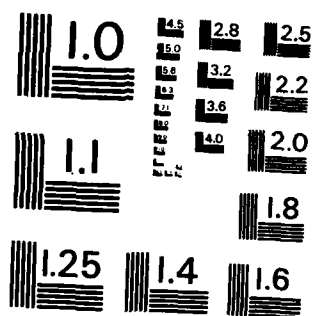
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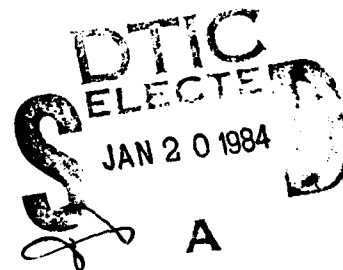
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DEVELOPMENT OF AN IN VIVO ASSAY FOR MISTRANSlation-  
INDUCING ACTIVITY OF POLLUTANTS AND CHARACTERIZATION OF AMINO  
ACID SUBSTITUTIONS

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into the cells being studied (see attached preprint). The above results were obtained using purified 0.3 protein. Additional experiments directed toward the first objective have led to a second procedure for quantitating cysteine misincorporation into 0.3 protein. A radioimmune precipitation (RIP) assay was developed which used polyclonal antibodies to 0.3 protein, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and scanning densitometry. We are currently preparing monoclonal antibody to 0.3 protein to obviate the need for SDS-PAGE and scanning densitometry. Experiments directed toward the second overall objective have provided interesting preliminary results. Trypsinization of cysteine-labeled 0.3 protein analysis of fragments by SDS-PAGE have shown that new peptide fragments are produced. This indicates that cysteine is substituting for arginine. Cleavage of cysteine-labeled 0.3 protein with CNBr and analysis of the peptide fragments by gel filtration, however, results in 3-4 labeled fractions which should not occur if cysteine were substituting only for arginine. This indicates that cysteine substitutes for at least one other amino acid besides arginine. These results will be confirmed and refined by HPLC (high performance liquid chromatography).

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## PROGRESS REPORT

Grant # AFOSR 81-0087

Title: Development of an in vivo assay for mistranslation-including activity of pollutants and characterization of amino acid substitutions

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### REPORT FOR RESEARCH UNDERTAKEN BETWEEN AUGUST 1, 1982 - JULY 31, 1983

#### I. Research Undertaken Between August 1, 1982 - February 1, 1983

##### A. Abstract

##### B. Details of experiments completed between 8/1/82 and 2/1/83

1. Determination of the error level in 0.3 protein translation by <sup>35</sup>S-cysteine misincorporation
2. Demonstration that <sup>35</sup>S-cysteine is not converted to <sup>35</sup>S-methionine

#### II. Research Undertaken Between February 1, 1983 - July 31, 1983

##### A. Overall objectives

##### B. Precise objectives for the period 2/1/83 - 7/31/84

##### C. Abstract

##### D. Details of experiments completed between 2/1/83 and 7/31/83

1. Progress in preparation of monoclonal antibody to the 0.3 protein
2. Experiments to determine whether cysteine substitutes only for arginine during 0.3 protein synthesis under normal (non-drug, non-pollutant) conditions

#### III. Manuscripts Submitted for Publication

A. Cloning using bacteriophage SPPlv as the vector: Vector development, stability, and expression, A.I. Deamyer, J.B. Rice, J.N. Reeve.  
(In Press.)

B. Mistranslation of a T7 bacteriophage protein, J.B. Rice, R. T. Libby, J.N. Reeve (Submitted to J. Biol. Chem.)

#### IV. Professional Personnel

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Chief, Technical Information Division

## **I. Report for research undertaken between August 1, 1982 - February 1, 1983**

### **A. Abstract**

As part of our efforts to develop a simple *in vivo* assay for the ability of pollutants to increase mistranslation, we have determined the normal frequency of mistranslation resulting in misincorporation of amino acids into the T7 0.3 protein. An average value of 0.019 cysteine residues per molecule was found in this non-cysteine-containing protein. Since there are 116 amino acids in the protein, this represents 1 cysteine misincorporated per 5,000 amino acids: a mistranslation frequency of  $3 \times 10^{-3}$  per codon. Control experiments were carried out in which the number of methionine residues in 0.3 protein was measured. Our experimental results indicated that 0.3 protein has 5.7 methionine residues per molecule. The DNA sequence indicates that 0.3 protein actually contains 6 methionines per molecule.

Additional evidence to support our value for normal mistranslation in 0.3 protein was the demonstration that  $^{35}\text{S}$  is not transferred from cysteine to methionine. Experiments using a methionine-cysteine double auxotrophic strain, *E. coli* KL266, gave values for methionine and cysteine incorporation into 0.3 protein similar to those obtained with the prototrophic *E. coli* strain B. Autoradiograms of thin layer chromatograms of material produced by hydrolysis of  $^{35}\text{S}$ -cysteine-labeled 0.3 protein showed that the radioactivity was still in cysteine and had not been metabolized to methionine.

### **B. Details of experiments completed between 8/1/82 and 2/1/83**

#### **1. Determination of the error level in 0.3 protein translation by $^{35}\text{S}$ -cysteine misincorporation**

Our last progress report described preliminary experiments which determined conditions for calculations of misincorporation of cysteine into the non-cysteine-containing T7-encoded 0.3 protein. Based on these experiments we have determined the mistranslation frequency during normal T7 infection of *E. coli* (Table 1). In similar experiments substituting  $^{35}\text{S}$ -methionine for  $^{35}\text{S}$ -cysteine, and adding  $2 \times 10^{-4}$  M methionine to saturate the methionine transport system, we have experimentally determined the number of methionine residues per 0.3 molecule (Table 1). The latter experiments

represent an ideal control for all parameters of the experiments since it is known (1) that the 0.3 protein contains 6 methionine residues.

Calculations used to determine the number of cysteine (or methionine) residues incorporated into the 0.3 protein are as follows:

- (1) Specific activity of  $^{35}\text{S}$ -cysteine (total number of cys residues/CPM) = 
$$\frac{\text{No. molecules } ^{35}\text{S-cys} + \text{No. molecules unlabeled cys in culture}}{\text{CPM of } ^{35}\text{S-cysteine added to culture}}$$
- (2) Specific activity of 0.3 protein (Number cys residues per molecule 0.3 protein) = 
$$\frac{\text{CPM of 1 ml of 0.3 protein} \times \text{specific activity of } ^{35}\text{S-cys}}{\text{molecules of 0.3 protein in 1 ml}}$$

The calculation requires labeling of 0.3 protein with  $^{35}\text{S}$  cysteine followed by biochemical purification of the protein and radiochemical measurements on the purified protein. The following scheme has been developed to purify the 0.3 protein and obtain the required measurements:

*E. coli* B is grown to  $8 \times 10^8$  cells/ml ( $A_{580} = 0.8$ ) (exponentially growing cells which are most susceptible to phage infection.)

↓  
Cells are washed twice in M9 minimal medium.

↓  
Cells are resuspended at  $2 \times 10^9$ /ml in 50 ml M9 minimal medium containing  $2 \times 10^{-4}$  M cysteine + 0.1 mg methionine/ml + 10% (v/v) Cysteine Assay Medium (Difco) (for  $^{35}\text{S}$ -cysteine labeling) or  $2 \times 10^{-4}$  M methionine + 10% (v/v) Methionine Assay Medium (Difco) (for  $^{35}\text{S}$ -methionine labeling).

↓  
The T7 mutant H31am193LG3 is added at MOI = 3.

↓  
 $^{35}\text{S}$ -cysteine (or  $^{35}\text{S}$ -methionine) is added at 10  $\mu\text{Ci/ml}$ .

↓  
Infected cells are incubated for 1 hr at  $37^\circ\text{C}$  with shaking.

↓  
Cells are centrifuged for 10 min at  $10,000 \times g$ .

↓  
Cells are resuspended in 2 ml lysis buffer + 20  $\mu\text{l}$  2 mg lysozyme/ml + 10  $\mu\text{l}$  0.5 mM PMSF (protease inhibitor).

↓  
Cells are incubated 30 min at  $32^\circ$ , then frozen and thawed 5 times.

↓  
DNA is digested with 10  $\mu\text{l}$  2 mg DNase/ml + 20  $\mu\text{l}$  1 M  $\text{MgSO}_4$  + incubation for 30 min at  $37^\circ\text{C}$ .



The volume is measured and  $\text{NH}_4\text{Cl}$  is added to 0.3 M.

↓  
The liquid is centrifuged for 15 min at 17,000 x g.

↓  
The clarified supernatant is passed through a DEAE column equilibrated with 0.3 M  $\text{NH}_4\text{Cl}$  - 20 mM Tris buffer, pH 8. The column (volume = 10 ml) is washed with 100 ml of 0.3 M  $\text{NH}_4\text{Cl}$ -Tris buffer. Acidic proteins are eluted with a 40 ml 0.3 M - 1.0 M  $\text{NH}_4\text{Cl}$ -Tris buffer gradient. Fractions (3 ml) are collected.

↓  
Fractions are tested for 0.3 protein by the Ouchterlony immunodiffusion technique using rabbit anti-0.3 antiserum, raised as part of this project.

↓  
Fractions containing the 0.3 protein are pooled, and protein is precipitated with 10% (w/v) trichloroacetic acid (TCA).

↓  
The precipitate is dissolved in Tris buffer, pH 8.8. The volume of buffer added is determined by the  $A_{280}$  sum of the pooled fractions. (Example: if the  $A_{280}$  sum is 4.0, 0.40 ml of buffer is added.)

↓  
Four volumes of 95% ethanol are added, and the solution is placed on ice for 30 min.

↓  
The solution is centrifuged for 15 min at 17,000 g.

↓  
The pellet is discarded because 0.3 protein is soluble in ethanol. An equal volume of 10% TCA is added to the supernatant.

↓  
The solution is placed on ice for 1 hr.

↓  
The solution is centrifuged for 15 min at 17,000 x g.

↓  
The pellet is dissolved in 0.5 ml Tris buffer, pH 8.8.

↓  
The pellet is tested for purity of 0.3 protein by SDS-PAGE (10-20% gradient) and stained for protein with the silver nitrate staining technique.

↓  
The quantity of pure 0.3 protein is determined using the Bio-Rad Protein Assay kit.

10  $\mu$ l of pure 0.3 protein is applied to filters (in triplicate) and counted in a scintillation counter. 5  $\mu$ l of a 1:1000 dilution of the  $^{35}\text{S}$ -cysteine (as supplied by the manufacturers) is also counted in triplicate.



Calculations are then performed.

2. Demonstration that  $^{35}\text{S}$ -cysteine is not converted to  $^{35}\text{S}$ -methionine

If  $^{35}\text{S}$  were transferred from cysteine to methionine, the calculated error level in the synthesis of 0.3 protein would be higher than the actual error level since methionine is present in 0.3 protein. We demonstrated  $^{35}\text{S}$  was not transferred from cysteine to methionine in two ways.

- (1) The hydrolysate of 0.3 protein, synthesized in the presence of  $^{35}\text{S}$ -cysteine, was resolved into individual amino-acid spots by thin layer chromatography (cellulose matrix using N-propanol: ammonium hydroxide (70:30) as solvent). Autoradiography was used to locate  $^{35}\text{S}$  on the chromatograms. The radioactivity was located in the cysteine spot and not in the methionine spot.
- (2) An *E. coli* strain auxotrophic for methionine and cysteine was used as host for T7 infection. (*E. coli* KL266). This strain is defective in *met E* (tetrahydropteroyltriglutamate methyltransferase) and *cys G* (adenylsulfate kinase) and cannot convert cysteine to methionine. The levels of  $^{35}\text{S}$ -cysteine incorporation into 0.3 in T7-infected *E. coli* KL266 were not significantly different from the levels of incorporation found with prototrophic *E. coli* strains (Table 1).

Table 1. Incorporation of Cysteine and Methionine into the 0.3 Protein

Experiment	<u>E. coli</u> strain <sup>1</sup>	No. cys residues <sup>2</sup> per molecule	No. met residues <sup>2,3</sup> per molecule
6	B	.021	—
7	B	.022	—
9	B	.028	—
11	B	.023	—
10	KL266	.012	—
13	KL266	.016	—
3	B	—	5.2
5	B	—	6.4
4	KL266	—	3.7
8	KL266	—	4.3

<sup>1</sup> E. coli B is prototrophic.

E. coli KL266 is F<sup>-</sup>, leu B6, proC32, hisF80, cysC43, thyA54, metE70, chi-1, ara-14, lac236, xyl-5, mtl-1, malA38, rpsE2115, (= spc A15), rpsL109 (= strA109).

<sup>2</sup> Values calculated on the basis of <sup>35</sup>S-cysteine or <sup>35</sup>S-methionine incorporation.

<sup>3</sup> Direct amino acid sequencing and the known DNA sequence predict 6 methionines per molecule of 0.3 protein (1).

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6	B	.021	—
7	B	.022	—
9	B	.028	—
11	B	.023	—
10	KL266	.012	—
13	KL266	.016	—
3	B	—	3.2
5	B	—	6.4
4	KL266	—	3.7
8	KL266	—	4.3

<sup>1</sup> E. coli B is prototrophic.

E. coli KL266 is F<sup>-</sup>, leu B6, proC32, hisF80, cysC43, thyA54, metE70, thi-1, ara-14, lac236, xyl-5, mtl-1, malA38, rpsE2115, (= spc A15), rpsL109 (= strA109).

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<sup>3</sup> Direct amino acid sequencing and the known DNA sequence predict 6 methionines per molecule of 0.3 protein (1).

## II. Research undertaken between February 1, 1983 and July 31, 1983

### A. Overall Objectives

1. To develop a simple, quantitative *in vivo* assay for the mistranslation-inducing activity of pollutants.
2. To determine the molecular basis for mistranslation resulting in the incorporation of cysteine into a protein which normally contains no cysteine.

### B. Precise objectives for the period 2/1/83 - 7/31/84

1. Production of a monoclonal antibody to the 0.3 gene product and use of this antibody in development of a radioimmune precipitation assay.
2. Direct determination of whether cysteine substitutes only for arginine in mistranslation during synthesis of 0.3 protein.

### C. Abstract

We have, in experiments directed toward the first overall objective, established the natural level of cysteine misincorporation into the bacteriophage T7 encoded 0.3 protein. We have also shown that this level can be increased by altering the environment of the translation machinery. This can be accomplished either by growing cells in the presence of mistranslation-inducing antibiotics or by introducing mutations which cause defective ribosomal proteins into the cells being studied (see attached preprint). The above results were obtained using purified 0.3 protein. Additional experiments directed toward the first objective have led to a second procedure for quantitating cysteine misincorporation into 0.3 protein. A radioimmune precipitation (RIP) assay was developed which used polyclonal antibodies to 0.3 protein, SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and scanning densitometry. We are currently preparing monoclonal antibody to 0.3 protein to obviate the need for SDS-PAGE and scanning densitometry. Experiments directed toward the second overall objective have provided interesting preliminary results. Trypsinization of cysteine-labeled 0.3 protein and analysis of fragments by SDS-PAGE have shown that new peptide fragments are produced. This indicates that cysteine is substituting for arginine. Cleavage of cysteine-labeled 0.3 protein with CNBr and analysis of the peptide fragments by gel filtration, however, results in 3-4 labeled fractions which should not occur if cysteine were substituting only for arginine. This indicates that cysteine substitutes for at least one other amino acid besides arginine. These results will be confirmed and refined by HPLC (high performance liquid chromatography).

### D. Details of experiments completed between 2/1/83 and 7/31/83

1. Progress in preparation of monoclonal antibody to the 0.3 protein

We have twice constructed hybridomas which produce the desired antibody but on both occasions the positive clones did not survive

continued serial passage. Problems of fungal contamination were also encountered. We are now into our second month of the third preparation, have no signs of fungal contamination, and we have several positive hybridomas. If these clones prove to be stable and we are successful in preparing pure monoclonal 0.3 protein antibody-producing clones, we will use this antibody in the RIP assay which we previously developed using polyclonal antibody. We anticipate that the pure antibody will precipitate only 0.3 protein ( $^{35}\text{S}$ -cysteine labeled) allowing quantitation of cysteine incorporation by simple scintillation counting of the immune precipitate.

2. Experiments to determine whether cysteine substitutes only for arginine during 0.3 protein synthesis under normal (non-drug, non-pollutant) conditions

Two types of experiments have been performed - trypsinization of  $^{35}\text{S}$ -cysteine-labeled 0.3 protein and chemical cleavage of  $^{35}\text{S}$ -cysteine-labeled 0.3 protein with CNBr. The 0.3 protein fragments were analyzed by SDS-PAGE (20% acrylamide) and by gel filtration (Sephadex G-50, Sephadex G-25, Bio-gel P6 and Bio-gel P4).

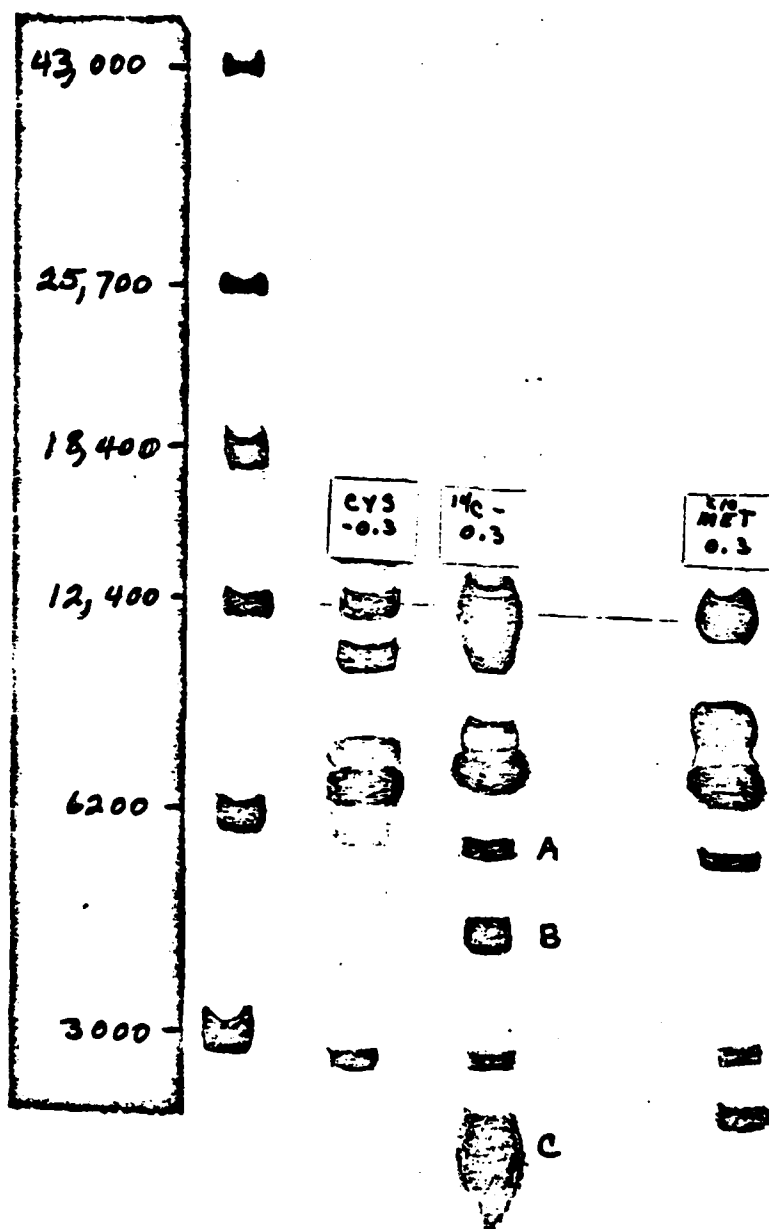
Analysis of 0.3 protein fragments by SDS-PAGE has been unsatisfactory because of lack of resolution of the lower MW peptide fragments. A smear is seen instead of discrete bands. We have been using the Laemmli "Tris discontinuous buffer system" with 20% acrylamide. A continuous sodium phosphate buffer system has been found to give somewhat better resolution of bands but the 0.3 peptide fragments migrate to different positions relative to the MW standards using this buffer system. Results of an experiment in which 0.3 protein was trypsinized and peptide fragments separated by SDS-PAGE using the sodium phosphate buffer system are shown in figure 1. The major peptide band of  $^{35}\text{S}$ -cys-,  $^{14}\text{C}$ -, and  $^{35}\text{S}$  met-labelled 0.3 proteins has an apparent MW slightly larger than 6000. Since the largest complete cleavage peptide should contain 47 amino acids, equivalent to a MW of 4700, this band is probably a partial cleavage peptide. It can be seen however, that whereas the major bands of  $^{35}\text{S}$  met- and  $^{14}\text{C}$ -labelled 0.3 protein have identical mobilities, that of  $^{35}\text{S}$  cys-labelled 0.3 protein is slightly faster. The bands marked "A", "B", and "C" are 47 amino acid, 32 amino acid, and 19 amino acid complete tryptic digestion products of 0.3 protein. There is no methionine in the 32 amino-acid fragment, and therefore this band does not show up in the lane containing  $^{35}\text{S}$  met-labelled 0.3 protein. The 19 amino-acid peptide (which contains methionine) can be seen on the original autoradiogram but is not easily seen on the photographic reproduction shown in Figure 1. Interestingly, there is no cysteine in the 32 amino acid peptide, whereas cysteine is found in the 19 amino acid peptide and in a smaller MW peptide.

There are six amino acids for which cysteine could possibly substitute by misreading of a single base in the codon: arginine, tyrosine, tryptophan, serine, phenylalanine, and glycine. From the trypsinization experiments we can deduce the following: We can tentatively say that cysteine substitutes for arginine because a

band with a different MW is seen only when 0.3 protein is labelled with cysteine. Relative to the major band of  $^{14}\text{C}$ - and  $^{35}\text{S}$ -met-labelled 0.3 protein, this band has an increased mobility (decreased size) whereas if an arginine site were no longer available for cleavage by trypsin because of cysteine substitution for arginine the cysteine-containing tryptic peptide should have an increased size. However, the possibility remains that this major  $^{35}\text{S}$ -cys-labeled band is the higher MW band predicted to be formed by lack of an arginine site N-terminal to the 47 AA peptide (band A). 2) The 32 amino acid peptide contains no cysteine (a result confirmed by gel filtration) and therefore cysteine is probably not substituting for tyrosine or tryptophan. (These are the only two amino acids of the possible six for which cysteine could substitute.) 3) Cysteine is found in the 19 amino acid peptide (determined by gel filtration) and therefore it appears that cysteine does substitute for serine and/or phenylalanine.

When cysteine-labelled 0.3 protein was cleaved with CNBr and fragments examined by gel filtration, similar results were observed. Figure 2 shows a typical experiment. 0.3 protein labeled with  $^3\text{H}$ -isoleucine or with  $^{35}\text{S}$ -cysteine were mixed, cleaved with CNBr, and analyzed on Sephadex G-50. If cysteine were substituting only for arginine, as suggested by Edelman and Gallant (Cell, 10:131-137, 1977) there should be peaks corresponding only to the 45 and 22 amino acid peptides (the only CNBr fragments which contain arginine), and any peptides resulting from partial digestion which contain one or both of these fragments. Peaks B, C, D and F contain peptides produced by partial digestion. Also seen are the 45 amino acid peptide (peak E) and the 22 amino acid peptide (peak G). Three additional peaks are however present (H, I, and J) and these correspond to peptides which do not contain arginine. Of the 6 amino acids for which cysteine could most likely substitute, these fragments instead contain tyrosine, phenylalanine, and serine; serine, glycine, and phenylalanine; and serine, respectively. Very similar peak profiles were obtained when peptide fragments were analyzed by Sephadex G-25, and Bio-gel P6 or P4. Our experiments to date therefore suggest that in mistranslation of the T7 0.3 protein, cysteine can at least substitute for arginine, and probably also for serine. Substitutions for other amino acids are, of course, still possible. Improved separation and identification of peptide fragments of 0.3 protein is needed before the precise substitution pattern can be obtained. We therefore plan to use high pressure liquid chromatography (HPLC) to better separate peptide fragments. In addition, we plan to include the use of reagents to cleave the 0.3 protein at other residues to confirm our present results.

FIGURE 1





4



**CLONING USING BACTERIOPHAGE SPPIV AS THE VECTOR:  
VECTOR DEVELOPMENT, STABILITY AND EXPRESSION<sup>1</sup>**

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**I. INTRODUCTION**

x S Bacteriophage SPPIV is a derivative of the virulent phage SPPI developed as a cloning vector (Heilmann and Reeve, 1982). Cloning is accomplished by insertion of DNA into the single BamHI site of the vector. Digestion of SPPIV vector DNA by BamHI reduced<sup>5</sup> the frequency of transfection of competent cells of Bacillus subtilis by approximately 1,000-fold. Cloning of restriction fragments generated by the enzymes BglII or BclI into the BamHI site of SPPIV creates recombinant molecules which, if the cloned DNA fragment does not itself contain an internal BamHI site, are not inactivated in transfection by exposure to BamHI. This provides a mechanism to selectively isolate recombinant phage from a ligation mixture; exposure of the ligation mixture to BamHI, following ligation and before transfection, prevents transfection by reconstituted SPPIV

<sup>1</sup>This research was supported by National Science Foundation Grant PCM 7912019 and contract 81-0087 from the Air Force Office of Scientific Research. J.N.R. is the recipient of Research Career Development Award AG00108 from the National Institutes of Health.

cloning  
A  
S  
A

X

vector phage. Cloning in SPPlv does not require expression of cloned DNA in infected B. subtilis cells nor does it require any homology between cloned DNA sequences and the B. subtilis chromosome. SPPlv is, in these respects, directly comparable to cloning in E. coli using a virulent  $\lambda$ -based vector and, as with  $\lambda$ , it is also possible to use plaque-hybridization procedure to directly identify recombinant phage carrying desired sequences. Plaque hybridization is particularly useful as a selection mechanism if BamH1 treatment of the ligation mixture is inappropriate, e.g. if BamH1 fragments themselves are to be cloned. Plaque hybridization was used, for example, to identify recombinant phage in which plasmid pUB110 had been cloned in SPPlv by ligation of BamH1 digested SPPlv to BamH1 digested pUB110 (Heilmann and Reeve, 1982). In this report, we describe construction of a derivative of SPPlv with increased cloning capacity, some unusual recombinants obtained in cloning using SPPlv and infection of minicells of B. subtilis to analyze the expression of SPPlv recombinant phages.

## II. CONSTRUCTION OF SPPlvm

XS

A

SPPlv was constructed by in vitro insertion of a unique BamH1 site into a viable deletion mutant,  $\Delta X$ , of SPPl (Heilmann and Reeve, 1982). The BamH1 site was placed within a region of the SPPl genome known to be nonessential for phage growth, directly adjacent to the region of the genome deleted by  $\Delta X$ . A novel deletion mutant of SPPl,  $\Delta M$ , was recently isolated in the laboratory of T. A. Trautner and generously provided to us for use in SPPl vector constructions. This deletion removes approximately 1 Kbp of DNA and, in so doing, deletes the thirteenth smallest EcoRI fragment of SPPl and fuses the eighth and tenth smallest EcoRI fragments (Figure 1). We have introduced  $\Delta M$  into SPPlv to create SPPlvm. DNA from SPPl $\Delta M$  was digested with BglI and ligated to the smallest BglI fragment of SPPlv prepared by extraction from an agarose gel following BglI digestion of SPPlv. This BglI fragment of SPPlv contains the BamH1 cloning site of SPPlv (indicated by the large arrowhead in Figure 1) and  $\Delta X$ . The presence of  $\Delta X$  in an SPPl genome results in a distinctive plaque morphology so that, following ligation and transfection with the mixture of SPPl $\Delta M$  DNA and the BglI fragment of SPPlv, phage from plaques with the  $\Delta X$  morphology were isolated. The structures of the genomes of these phage were analyzed by agarose gel electrophoresis. The patterns of EcoRI restriction fragments of SPPlw.t. (wild-type), SPPl $\Delta M$ , SPPlv

## Figure 1

and SPPlvm are shown in Figure P01. It can be seen that SPPlvm contains both  $\Delta X$  (reducing the size of EcoRI fragment 1) and  $\Delta M$  (fusing EcoRI fragments 8 and 10 and deleting EcoRI fragment 13). The net deletion of DNA in SPPlvm is approximately 4.1 Kbp. SPPlv.t. phage particles normally package approximately 1.8 Kbp of terminally redundant DNA. There should therefore be sufficient space in SPPlvm to allow the cloning of at least 5.9 Kbp of foreign DNA into the BamHI site of this vector. All procedures developed for use with SPPlv are equally applicable to SPPlvm and the presence of  $\Delta M$  provides a physical location marker for orientation and measurement of molecules formed in DNA:DNA heteroduplex experiments.

### III. DELETIONS OBSERVED IN CLONING WITH SPPlv

During the development of SPPlv and in subsequent use of SPPlv to clone specific DNA fragments we have frequently isolated recombinant phage which do not contain precisely the fragment of DNA introduced into the ligation reaction. Many recombinant phage appear to have suffered major deletions and in some cases, the vector has undergone major genomic reorganizations (Desmyter, Heilmann, Reeve, Morelli and Trautner, in preparation). Similar anomalous results have also been reported by Behrens *et al.* (1983) using the cloning vehicle SPPlvic which is very similar to SPPlv but contains a unique PstI cloning site rather than a BamHI site. We have undertaken a series of experiments to further investigate this phenomenon. A recombinant plasmid, pET407, which consists of DNA from the methanogenic microorganism Methanobrevibacter smithii and most of the sequences of plasmid PBR322 (Hamilton and Reeve, 1983) was used as the test substrate for cloning into SPPlv. The location and origin of DNA in recombinant phage, which had originally been part of pET407, were determined by restriction enzyme analyses and by Southern blot analyses. Radioactive probes specific for either the methanogen or pBR322 derived DNA sequences were used. Results from one series of experiments are shown in Figure 2. SPPlv and pET407 were digested with BamHI, ligated together, and following transfection phage were isolated from plaques which gave a positive signal using  $^{32}P$ -labeled pET407 as the probe in a plaque hybridization screen. Recombinants were not found which contained the complete sequence of pET407. Analyses identified phage in which deletions had occurred either in the vector and/or in the pET407 sequences. In some cases more than one phage was present in the preparation and by

SPET2 X  
repeated plaque purification it was possible to show that a larger recombinant phage was continuously giving rise to a smaller phage by deletion. An example is shown in Figure 2 in which phage SPET21 was routinely produced by growth of SPET2 in B. subtilis. Many phage were isolated which, even though they gave an initial positive reaction to the pET407 hybridization probe, were subsequently found to be identical to SPP1v (not shown in Figure 2). Construction of SPP1v created a situation in which cloned fragments are flanked by a directly repeated DNA sequence at least 10 base pairs in length (Heilmann and Reeve, 1982). Excision of cloned fragments may be occurring by recombination at the BamH1 sites at each end of a cloned fragment facilitated by the extensive region of homology extending from these BamH1 sites.

λ  
Experiments to date have necessarily employed recombination proficient strains of B. subtilis because SPP1 transfection of competent cells requires Rec<sup>+</sup> cells (Trautner and Spatz, 1973). Experiments are currently in progress to determine if the frequency of deletion of occurrence is less using transfection of either Rec<sup>+</sup> or Rec<sup>-</sup> protoplasts. Unfortunately SPP1 does not form plaques on lawns of B. subtilis grown on the standard medium used for protoplast regeneration and this technical problem must first be resolved. The results typified by the SPET2 → SPET21 observations (Figure 2) do demonstrate, however, that not all of the deletions observed following transfection of competent cells are directly attributable to the processing that DNA undergoes during transfection. As previously observed for plasmid based recombinant molecules in B. subtilis (Kreft et al., 1982), there also appear to be preferred sites for deletion events to occur in B. subtilis based recombinant molecules during their replication in B. subtilis cells.

in SPP1v-based

#### IV. CLONING THE 0.3 GENE OF COLIPHAGE T7 IN SPP1v

SPP1v X  
We are currently investigating the fidelity of synthesis of the product of the 0.3 gene of coliphage T7 in infected cells of E. coli by determining the frequency of misincorporation of <sup>35</sup>S-cysteine into this protein which normally does not contain cysteine residues (Rice, Libby and Reeve, manuscript submitted for publication). To extend this work to the fidelity of 0.3 protein synthesis in B. subtilis, it was necessary to clone the 0.3 gene in SPP1v. Several previous attempts to clone the intact early region of T7, either in E. coli (Studier and Rosenberg, 1981) or B.

have been

^ subtilis (Scherzinger et al., 1980), were unsuccessful. We were also unable to clone the complete early region of the T7 genome into SPPlv (H. Heilmann, A. Desmyter and J. Reeve, unpublished results). We therefore cloned the 0.3 gene into SPPlv starting from the plasmid, pAR324 (provided by F.W. Studier (Studier and Rosenberg, 1981)), which consists of the 0.3 gene cloned in the BamH1 site of pBR322. BamH1 digested SPPlv was ligated to the fragment of DNA containing the 0.3 gene isolated by agarose gel electrophoresis from a BamH1 digest of pAR324. Following transfection, <sup>32</sup>P-labeled pAR324 DNA was used as a hybridization probe to identify plaques containing SPPlv::0.3 recombinant phage. Phage were obtained with the T7 0.3 gene sequence in either orientation. Phage with the 0.3 gene in the correct orientation for transcription starting from the SPPl promoter 2 (Stüber et al., 1981) (opposite to the normal orientation for T7 transcription) were designated SPPlv::0.3+ and those with the incorrect orientation for transcription were designated SPPlv::0.3-. Figure 3 shows the details of the 543 bp fragment of the T7 genome cloned into SPPlv. The asymmetric AccI site was used to determine the orientation of cloning. This site may, itself, be useful in cloning as it is located immediately following the initiation ATG codon for the T7 0.4 gene and is preceded by translation stop signals and an apparently good ribosome binding sequence (Dunn and Studier, 1981). The diagram illustrates that there are sequences preceding the 0.3 and 0.4 genes, 5 and 6 bases in length, respectively, which as mRNAs should hybridize to the 16S rRNA of both E. coli and B. subtilis ribosomes.

#### V. EXPRESSION OF SPPlv RECOMBINANT PHAGES IN INFECTED MINICELLS

Minicells of B. subtilis were infected with recombinant phage and allowed to incorporate <sup>35</sup>S-methionine. Radioactively labeled polypeptides, synthesized in infected minicells, were analyzed by fluorography following polyacrylamide gel electrophoresis as previously described for analysis of SPPl gene products (Mertens et al., 1979). We are unable to detect differences between the polypeptides synthesized in SPPlv recombinant phages. There are SPPl-encoded polypeptides which migrate, during electrophoresis, to approximately the same location as the 0.3 protein (see Figure 4). We have therefore used rabbit anti-0.3 antiserum in attempts to precipitate radioactively labeled 0.3 protein from SPPlv::0.3+ infected minicells to ensure that co-

meat \* infected minicells and those synthesized in minicells infected by a variety of SPPlv

SPVW  
cells  
migration of polypeptides was not preventing detection of 0.3 protein synthesis. The antiserum did not precipitate radioactively labeled 0.3 protein confirming that this protein is not synthesized in detectable amounts in SPP1v::0.3+ minicells of B. subtilis. Minicells infected with SPP1v::0.3+ were allowed to incorporate 5,6[<sup>3</sup>H]-uridine to label mRNA. The labeled mRNA was used in RNA:DNA filter binding hybridization experiments. As shown in Table 1, very few, if any, transcripts of the 0.3 gene were present in the RNA preparation. It must therefore be concluded that the cloned 0.3 gene is not efficiently transcribed in infected minicells even though the gene is cloned in a region of the SPP1 genome which is heavily transcribed in SPP1 infected cells (Stüber et al., 1981) and which was previously shown to be expressed in infected minicells (Mertens et al., 1979). We are currently determining whether the 0.3 gene is expressed in SPP1v::0.3+ infected nucleated cells of B. subtilis.

## VI. CONCLUSIONS

SPP1v and SPP1vm can be used to clone and amplify specific fragments of DNA in B. subtilis without the need for gene expression. As with plasmid cloning in B. subtilis, there are frequent problems of instability of recombinant genomes when SPP1v is used as the vector, but as shown by the cloning of the T7 0.3 gene, it is also not difficult to obtain desired recombinants. SPP1v, SPP1vm and SPP1vic (Behrens et al., 1983) are virulent phages and as such are not ideal for use as expression systems. Phage infection of minicells may be used to investigate encoded mRNAs and polypeptides (Reeve, 1979). There are, however, significant differences between SPP1 expression in nucleated cells and in minicells (Mertens et al., 1979) and there are most probably major differences in the quantitative expression of genes cloned in SPP1 vectors in infected cells and in infected minicells. The methyl transferase gene cloned by Behrens et al. (1983) in SPP1vic is clearly functionally expressed in infected B. subtilis cells but there is no discernible difference between the polypeptides synthesized in minicells infected by the vector, SPP1vic, and the polypeptides synthesized in minicells infected by the SPP1vic-methyl transferase encoding recombinant (J. Reeve and T. Trautner, 1983; unpublished results). Phage infection does offer the opportunity of introducing recombinant DNA molecules into B. subtilis cells without the need for either competent cells or protoplasts. Infection

can therefore be used to study immediate effects of cloned genes on actively growing, physiologically normal cells. Incorporation of appropriate mutations into the viral vector genome should create a situation in which viral genes are not expressed and infected cells are therefore not immediately killed.

All phage described in this report have been deposited in the Bacillus Genetics Stock Center for general distribution.

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Figure 1. Construction of SPPlvm. The EcoRI restriction map of SPPlw.t. is shown above the BglI restriction map of SPPlv. The location of  $\Delta X$  and  $\Delta M$  are indicated. The single BamHI site introduced into SPPlv (Heilmann and Reeve, 1982), also present in SPPlvm is indicated by the heavy vertical arrowhead. The right of the figure shows agarose gel electrophoretic separations of the



X EcoRI restriction fragments (with numerical designations to the left of the gel) produced by digestion of the genomic DNAs of the phage listed above each track.

ix  
^  
x 4 ^  
SPET2 x  
an  
^  
B,  
^  
Figure 2. Physical structure of the DNA of recombinant phage obtained in cloning pET407 in SPPlv. The top line in the diagram depicts pET407 linearized by BamH1 digestion. The broken line is M. smithii DNA and the solid line pBR322 DNA (Hamilton and Reeve, 1983). A restriction map of the DNA adjacent to the BamH1 site in SPPlv, also cleaved at the BamH1 site, is shown directly below the pET407 map. The physical structure of a series of recombinant phage, designated SPET1, SPET2, etc. obtained in an attempt to clone pET407 into SPPlv are shown below SPPlv. Deleted areas of the vector are indicated by large dots and deleted areas of pET407 by small dots. SPET21 arose spontaneously during growth of SPET2 in B. subtilis MCB. SPET5 contains a small but undefined fragment of pBR322 DNA as shown by Southern blotting. Sites for the enzymes EcoRI, BamH1, Avall, HindIII and BglI are indicated by the symbols E, A, H, and Δ, respectively. There are no differences in the regions of the genomes of the phages which are not shown in the Figure.

X-pair  
in as  
are  
Figure 3. Structure of the T7 DNA, containing the T7 0.3 gene, cloned in SPPlv. Plasmid pAR324 (Studier and Rosenberg, 1981) consists of pBR322 plus the fragment of T7 DNA shown in the diagram. The T7 fragment was cloned in the BamH1 restriction site of pBR322 using BamH1 linkers. Figures below the DNA fragment are the location of that particular base-pair in the T7 genome designating the extreme left end of T7 and 0. All details of T7 in the figure are based on the results presented by Dunn and Studier (1981).

meth x  
^  
4  
Figure 4. Autoradiogram of the electrophoretic separation of radioactively labeled polypeptides synthesized in minicells. Minicells were infected by different recombinant phage, indicated above the tracks, and allowed to incorporate <sup>35</sup>S-methionine. Radioactively labeled polypeptides were separated by electrophoresis through a 10 to 20% polyacrylamide gel which was dried and used in fluorography (Reeve, 1979). Purified, <sup>35</sup>S-labeled polypeptides synthesized in E. coli minicells containing pBR322 and pET405 were run as standards. E. coli minicells containing pBR322 and pET405 synthesize β-lactamase and E. coli minicells containing pET405 also synthesize two M. smithii polypeptides of molecular weights 53,000 and 37,000 (Hamilton and Reeve, 1983). The DNA encoding the 37,000 polypeptide is present in SPET2 but this polypeptide does

0.3 protein and  
<sup>35</sup>S-labeled  
insert

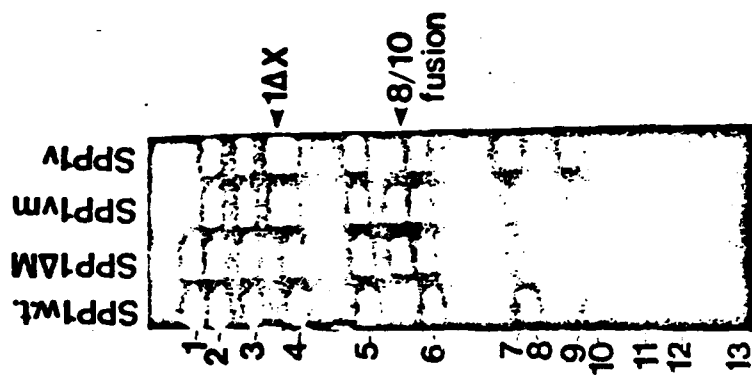
not appear to be synthesized in SPET2 infected minicells of  
B. subtilis.

Table 1. Hybridization of  $^3\text{H}$ -RNA Synthesized in SPP1v::0.3+ Infected Minicells to Filter-Bound DNA<sup>a</sup>

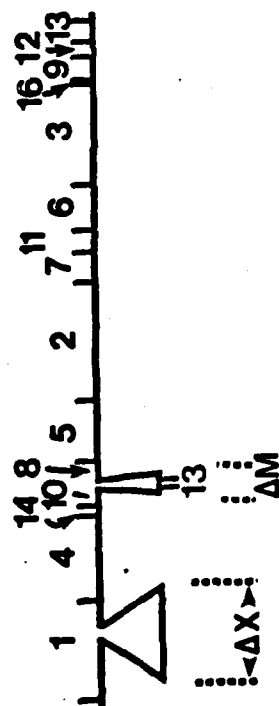
DNA on filter (5 $\mu\text{g}$ DNA/filter)	$^3\text{H}$ -RNA hybridized (c.p.m.)	% of input radioactivity bound
---	209	1.4
SPP1v	6454	44.2
pAR324	217	1.5
pBR322	192	1.3

<sup>a</sup>Minicells ( $4 \times 10^{10}$ ) from B. subtilis CU403 thyA thyB metB. divIVB1 (Reeve et al., 1973) were infected (input m.o.i.=1) with SPP1v::0.3+ as described by Mertens et al. (1979). The infected minicells were allowed to incorporate [5,6- $^3\text{H}$ ]-uridine (150  $\mu\text{Ci/ml}$ ; 47 Ci/mole; Amersham Corp., IL, 60005) for 30 minutes at 37°C. Radioactively labeled RNA was prepared and DNA:RNA hybridization carried out exactly as described by Miller (1972).

The fragment of T7 DNA cloned in SPP1v::0.3+ is approximately 1% of the total size of this genome. Assuming that all the genome were transcribed equally, transcription of the 0.3 sequence should have been clearly detectable by comparing the amount of  $^3\text{H}$ -RNA bound to pBR322 with the amount bound to pAR324. These plasmids differ only by the presence of the fragment of T7 DNA in pAR324 (see Figure 3 and Studier and Rosenberg, (1981)).



Net deletion  
in Kbp.



3.1



4.1



Figure 1.

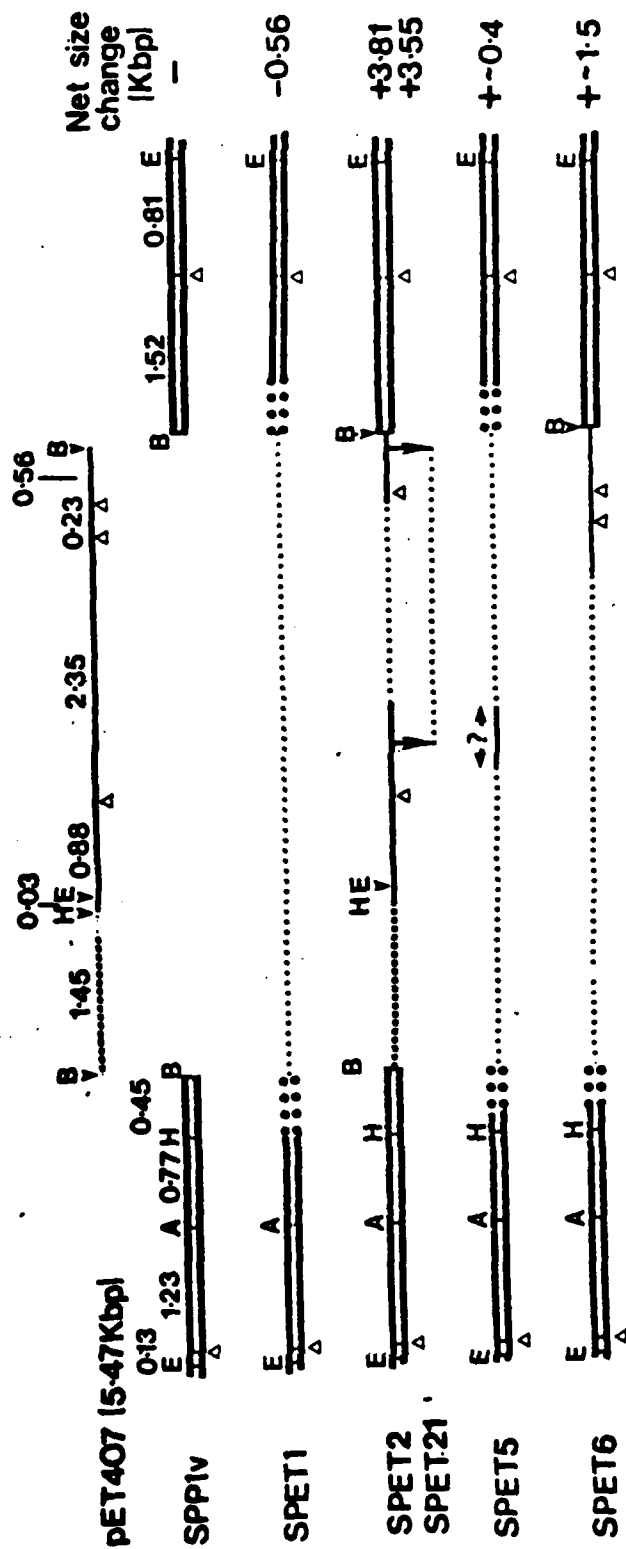


Figure 2

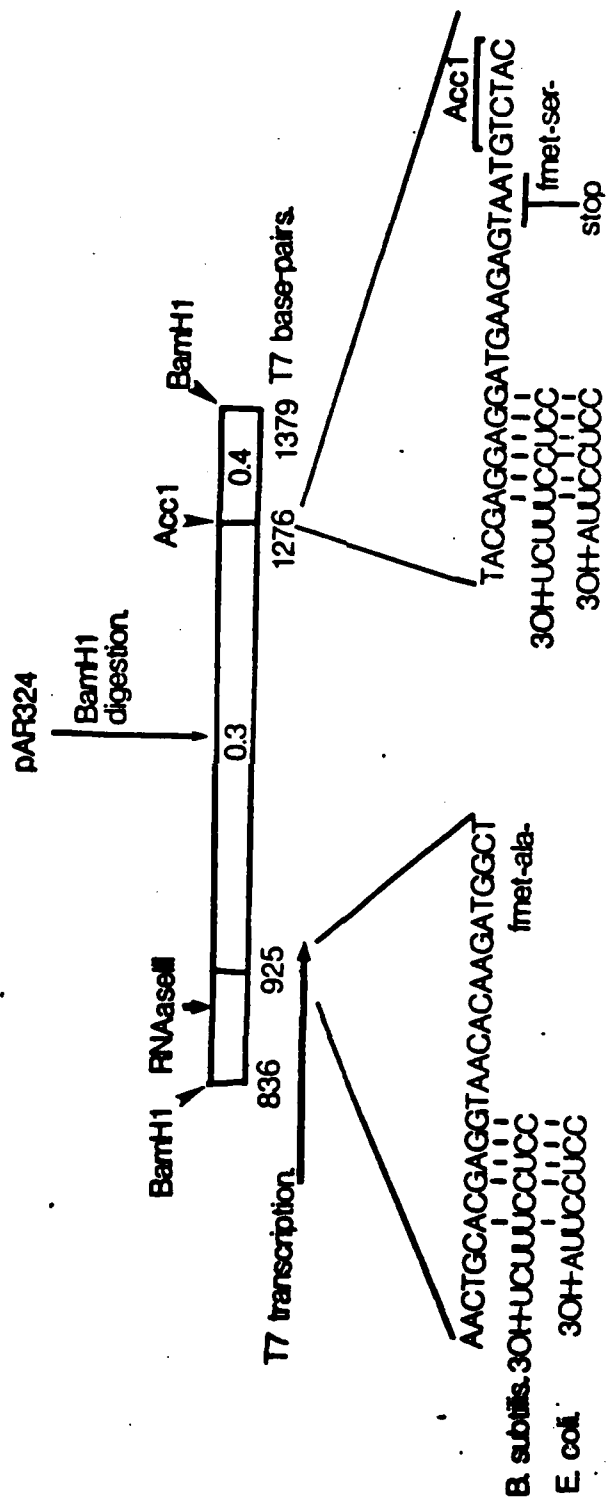


Figure 3

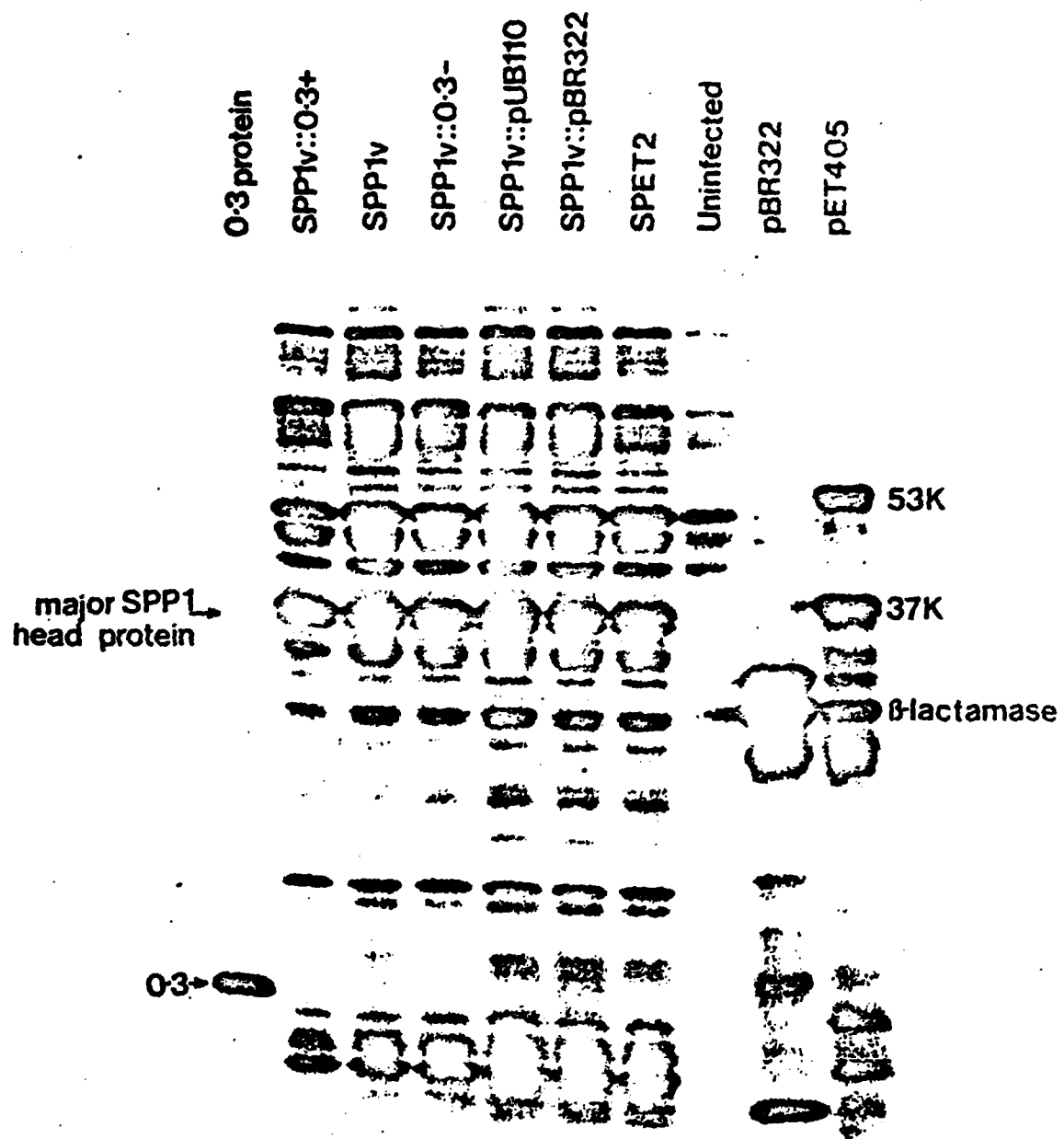


Figure 4

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<sup>1</sup>  
MS-2663

Title = Mistranslation of a T7 Bacteriophage Protein

(send proofs to Jacqueline B. Rice, Department of Microbiology, The Ohio State  
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Running title = Mistranslation of 0.3 Protein

#### Summary

We have devised an experimental system using the T7 phage 0.3 protein to accurately quantitate in vivo errors in translation. The 0.3 protein is well-suited for mistranslation studies because it is easy to purify, its entire amino acid and RNA sequences are known, and it contains no cysteine. Utilizing <sup>35</sup>S-cysteine as precursor we found an average of 1 cysteine misincorporated for every 43.5 molecules of 0.3 protein synthesized. Since there are 116 amino acids in 0.3 protein, one cysteine residue was misincorporated per 5000 codons translated. If all 20 amino acids were misincorporated at the same frequency, the general mistranslation level for 0.3 protein would be  $4 \times 10^{-3}$  per codon. Parallel <sup>35</sup>S-methionine incorporation experiments supported the accuracy of our findings for cysteine misincorporation. We found an average of 5.7 methionine residues incorporated per molecule of 0.3 protein synthesized, while the actual number from sequence data is known to be 6. Antibiotics which stimulate misreading (gentamicin and streptomycin) caused an increase in the number of cysteine residues misincorporated into 0.3 protein but the increase was modest. The use of isogenic *E. coli* strains, identical except for mutations in ribosomal protein genes known to affect fidelity of translation, supported the assumption that our system quantitates mistranslation rather than mistranscription.

## Introduction

Errors produced by a complex mechanism are necessarily characteristic of its normal modes of functioning. Thus, information about the translational process could be gained by studying errors in translation. Reports of the frequency of mistranslation in vivo in eukaryotic and procaryotic cells have been in the range of one error per  $10^3$ - $10^4$  correct amino acid insertions (1-9). Tests to measure the accuracy of protein synthesis in vivo are difficult to devise because the frequencies of mistranscription and mistranslation in vivo are very low and the protein or peptide of interest must be first separated from the total proteins and then accurately quantitated. Incorporation of an incorrect amino acid into a polypeptide has been detected by three different techniques. The first method is based on a charge difference between the correct and incorrect amino acid. Incorporation of the incorrect amino acid causes a change in the isoelectric point of the polypeptide which can be detected by two-dimensional gel electrophoresis (10). Only amino acid substitutions resulting in a peptide charge difference can be detected by this technique and it is usually not sensitive enough to detect the low levels of mistranslation in normally growing cells (11). Mistranslation can also be detected in nonsuppressing *E. coli* strains as synthesis of full length polypeptides encoded by genes which carry a nonsense mutation. Mistranslation of nonsense mutations has been detected by assaying functional  $\beta$ -galactosidase (7) and alkaline phosphatase (7, 12). The disadvantage of this technique is that only substitutions of amino acids which lead to an active protein will be detected and some amino acid substitutions may result in activities which are less than wild-type. The third method can be used when a technique is available to quantitatively purify a protein or peptide which is known not to contain a particular amino acid. Edelman and Gallant (4) measured the misincorporation of  $^{35}\text{S}$ -cysteine in non-cysteine-containing flagellin of *E. coli*. Purification

of labeled flagellin was facilitated by the extra-cellular location of this protein. Our experimental system has made use of the relatively easy purification of E. coli T7 phage 0.3 protein (13) and the fact that it also contains no cysteine (14). We have found misincorporation of cysteine into 0.3 protein to be 40 times higher than that observed for E. coli flagellin (4) but similar to the level of lysine for asparagine substitution in MS2 phage coat protein (determined by 2-dimensional electrophoresis) (15) and the mistranslation level of an E. coli ochre mutation of alkaline phosphatase (7).

### Experimental Procedures

Phage and Bacteria - The sources and description of *E. coli* strains used in this study are shown in Table 1. The mutations in bacteriophage T7,  $\Delta$  H3, 1am 193,  $\Delta$  LG3, are described by Studier et al., (16). *E. coli* cells infected with T7  $\Delta$  H3, 1am/193 $\Delta$  LG3 synthesize large amounts of the T7 0.3 protein and virtually no other T7 protein (13). T7  $\Delta$  H3, 1am 193,  $\Delta$  LG3 was grown in *E. coli* BBw/t. Phage were purified by ultracentrifugation in a CsCl step gradient and titrated on *E. coli* BBw/t. *E. coli* strains were constructed by transduction using P1vir according to the procedure of Miller (17).

Media and Antibiotics - Immediately before use *E. coli* cells were grown in LB complete medium. M9 minimal medium without sulfate supplemented with 1.0  $\mu$ g thiamine/ml and 1  $\mu$ M  $MgCl_2$  was used for all experiments. This medium was supplemented with 100  $\mu$ g thymine/ml for experiments employing *E. coli* KL266. Gentamicin sulfate and neomycin sulfate were obtained from Sigma Chemical Company and streptomycin sulfate from Eli Lilly and Company.

Production of Antiserum - New Zealand white rabbits were immunized intramuscularly with 0.3 protein (500  $\mu$ g in complete Freund's adjuvant) and 9 weeks later were boosted intradermally with 500  $\mu$ g 0.3 protein in incomplete Freund's adjuvant. The animals were bled 2-4 weeks later. The rabbit antiserum was shown to be specific for the 0.3 protein in radioimmune precipitation (RIP) experiments in which T7 CR10b, an amber mutant in gene 0.3 (18), was used to infect, in the presence of  $^{35}S$ -methionine, either *E. coli* BBw/t ( $Su^+$ ) or *E. coli* B ( $Su^0$ ). SDS-PAGE and autoradiographic analyses of the immune precipitate showed a heavy band in the 0.3 protein position only when *E. coli* strain BB w/t

was used (data not shown).

Radioimmune Precipitation (RIP) and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) - *E. coli* cells growing exponentially in complete LB medium were washed with M9 medium and resuspended to  $2 \times 10^9$  cells/ml in M9 medium containing 10% (v/v) methionine assay medium or cysteine assay medium (Difco). Cells (0.2 ml of suspension in Eppendorf tubes) were infected with T7 phage at an input m.o.i. of 5. For experiments shown in figure 1, minicells were prepared from *E. coli* RL002 and infected with T7 as previously described (19, 20).  $^{35}\text{S}$ -methionine or  $^{35}\text{S}$ -cysteine was added at 50  $\mu\text{Ci/ml}$ . Cells were incubated at 37°C for 30 min, 10  $\mu\text{l}$  of 20 mM  $\text{NaN}_3$  was added and the tubes centrifuged for 2 min in an Eppendorf model 5412 centrifuge. The cells were resuspended in 10  $\mu\text{l}$  50 mM Tris HCl, pH 8, 10 mM  $\text{Na}_3\text{EDTA}$ , and 4% (v/v) glycerol containing 10  $\mu\text{g}$  lysozyme. Following incubation at 37°C for 15 min, the cell suspensions were frozen and thawed 5 times. DNaseA (10  $\mu\text{g}$ ) and  $\text{MgSO}_4$  (10  $\mu\text{l}$  of 1.0 M) were added and the mixture incubated for 15 min at 37°C. NET buffer (200  $\mu\text{l}$  of 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.2% (w/v)  $\text{NaN}_3$ , 0.05% (v/v) NP40, 0.1% (w/v) bovine serum albumin, pH 7.4) was added and the tubes centrifuged for 2 min.. The supernatant (100  $\mu\text{l}$  aliquots) was transferred to 2 tubes. Antiserum (10  $\mu\text{l}$ ) was added to one tube and serum from a non-immunized rabbit (10  $\mu\text{l}$ ) was added to the other tube. The mixtures were incubated overnight at 4°C. Protein A Sepharose CL-4B (100 mg in 100  $\mu\text{l}$  NET buffer (Pharmacia)) was added to each tube and incubation at 4°C continued for 1 h. The tubes were centrifuged for 3 min in the Eppendorf centrifuge and the resulting pellet washed 5 times with NET buffer. SDS-PAGE sample buffer (stacking gel buffer containing 3% SDS, 5% 2-mercaptoethanol and 10% glycerol) (0.1 ml) was added to the washed pellets, the mixtures boiled for 3 min and then centrifuged for 3 min at room temperature. Polypeptides in aliquots of the supernatants were

separated by electrophoresis through 10-20% gradient SDS-PAGE slab gels and radioactively labeled polypeptides were detected by fluorography as previously described (21).

**0.3 Protein Purification** - A modification of the published procedure (13) was used. Exponentially growing *E. coli* cells in 4L LB complete medium were infected with a lysate of T7  $\Delta$ H3,  $\Delta$ lam193,  $\Delta$ LG3 at an input m.o.i. of 2-3. The infected cells were shaken for 60 min at 37°C. Cells were centrifuged at 7600 x g for 15 min at 4°C and resuspended in 20 ml 50 mM Tris-HCl, pH 8, containing 10 mM Na<sub>3</sub> EDTA, 4% glycerol and 40  $\mu$ g lysozyme. Cell lysis resulted from 5 cycles of freezing and thawing the cell suspension. DNaseA (300  $\mu$ g) and 300  $\mu$ l 1 M MgSO<sub>4</sub> were added, the mixture shaken for 30 min at 32°C, adjusted to 0.3 M NH<sub>4</sub>Cl and centrifuged at 17,000 x g at 4°C for 15 min. The supernatant fraction was centrifuged at 123,000 g at 4°C for 1 h and this supernatant was passed through a 10 ml DEAE-cellulose column (in a 50 ml disposable syringe), equilibrated with 0.3 M NH<sub>4</sub>Cl, 20 mM Tris HCl, pH 8, and 4% (v/v) glycerol. The column was washed with 150 ml of this buffer and proteins bound to the column were eluted with a 100 ml gradient of 0.3 M to 1 M NH<sub>4</sub>Cl in 20 mM Tris HCl, pH 8, 4% glycerol. Five ml fractions were collected and tested for 0.3 protein by 2-dimensional immunodiffusion using rabbit anti-0.3 antiserum. Fractions containing 0.3 protein were pooled and the proteins precipitated by addition of an equal volume of cold 10% (w/v) trichloroacetic acid (TCA). Following centrifugation (15 min at 4°C at 17,000 x g), the resulting pellet was dissolved in 0.3 M Tris HCl, pH 8.8, to give a solution of approximately 10 mg protein/ml. Four volumes of 95% ethanol were added and after 30 min on ice, the solution was centrifuged for 15 min at 4°C at 17,000 x g. The 0.3 protein is soluble in ethanol (13) and therefore remained in the supernatant. 0.3 protein was precipitated from the supernatant by addition of an equal volume of cold 10% (w/v) TCA, incubation for



at least one hour on ice and centrifugation at  $17,000 \times g$  for 15 min. The pellet of 0.3 protein was dissolved in a small volume (<sup>approximately</sup> 2 ml) of 0.3 M Tris HCl, pH 8.8, and tested for purity by electrophoresis through polyacrylamide gradient gels (21), and stained for proteins using the sensitive silver-based technique (22). Protein in solution was quantitated using a Bio-Rad Protein Assay kit and Bio-Rad bovine plasma albumin as a standard.

Cysteine and Methionine Incorporation Experiments - Exponentially-growing *E. coli* cells in LB complete medium were washed twice with M9 medium and resuspended in 50 ml M9 medium at  $2 \times 10^9$  cells/ml. CsCl-purified T7  $\Delta$  H3, 1 am 193,  $\Delta$  LG 3 was added at an m.o.i. of 2-3. For cysteine incorporation the 50 ml M9 minimal medium supplemented with 10% (v/v) Cysteine Assay Medium (Difco) also contained  $^{35}\text{S}$ -cysteine (New England Nuclear Corp.) at 10  $\mu\text{Ci/ml}$ , unlabeled cysteine at 200  $\mu\text{M}$ , and unlabeled methionine at 0.1 mg/ml (670  $\mu\text{M}$ ). For methionine incorporation experiments the 50 ml M9 minimal medium supplemented with 10% (v/v) methionine assay medium (Difco) also contained  $^{35}\text{S}$ -methionine (New England Nuclear Corp.) at 10  $\mu\text{Ci/ml}$  and unlabeled methionine and cysteine both at 200  $\mu\text{M}$ . Radioactively-labeled 0.3 protein was purified as described above using smaller amounts of reagents (usually 1/10 as much).

Thin Layer Chromatography - Purified 0.3 protein, synthesized in the presence of  $^{35}\text{S}$ -cysteine was hydrolyzed in 6N HCl at  $110^\circ\text{C}$  for 22 h using a sealed hydrolysis chamber. HCl was evaporated under vacuum and the hydrolysate resuspended in water. Unlabeled cysteine (12 ug) and methionine (15 ug) were added as markers and the mixture spotted on an Eastman cellulose chromatogram sheet (Eastman Kodak Company). Vertical thin layer chromatography was accomplished using N-propanol: 34%  $\text{NH}_4\text{OH}$  (7:3 ratio) as the solvent. Cysteine and methionine were located by ninhydrin staining. The chromatogram sheet was

used in autoradiography to locate  $^{35}\text{S}$ -cysteine and  $^{35}\text{S}$ -methionine. Locations of these compounds were compared to the ninhydrin determined locations of the non-radioactive cysteine and methionine.

### Results

The Experimental System - We chose the T7 0.3 protein (an anti-restriction protein) (18) as a tool for our mistranslation studies because it is produced in abundance after infection of *E. coli* with appropriate T7 mutants (13); infection acts as a zero time for synthesis and therefore all of the test protein is made during the labeling period; 0.3 protein is easily purified (13); the amino acid and RNA sequences are known (14), and it contains no cysteine residues. Figure 1 shows that there are, in fact, three T7 proteins which do not contain cysteine, namely the products of the early 0.3 gene and the late genes 9 and 16. The latter two are, however, made in much smaller amounts than the 0.3 protein.

In order to quantitate the incorporation of cysteine residues into 0.3 protein, it was necessary to know the specific activity of cysteine used by the cell in protein synthesis. To prevent dilution of the  $^{35}\text{S}$ -cysteine by endogenously synthesized cysteine, unlabeled cysteine was added at a known, saturating level (23). Excess unlabeled methionine was also provided to prevent cysteine conversion into methionine (24). The cysteine assay medium provided all the other amino acids and this prevented cysteine catabolism to pyruvate (23). Dilution experiments showed that the minimum saturating level for both cysteine and methionine utilization in *E. coli* B was 200  $\mu\text{M}$ . An increase in cysteine or methionine concentration above this level did not result in any additional cysteine or methionine incorporation into proteins (data not shown).

T7 infected cells were allowed to incorporate  $^{35}\text{S}$ -cysteine or  $^{35}\text{S}$ -methionine. They were then lysed and 0.3 protein was purified in 2 steps: DEAE cellulose anion exchange chromatography and extraction of the eluate with 95% ethanol (see Experimental Procedures). Figure 2 is a photograph of a silver-stained SDS-PAGE gel showing the purity of the 0.3 protein following the ethanol extraction step.

To calculate the number of cysteine (or methionine) residues incorporated per molecule of 0.3 protein synthesized, it was necessary to determine the specific activity of the purified 0.3 protein and of the  $^{35}\text{S}$ -cysteine (or  $^{35}\text{S}$ -methionine) precursor provided. The latter value was determined by scintillation counting of a known aliquot of the  $^{35}\text{S}$ -cysteine (or  $^{35}\text{S}$ -methionine) preparation and dividing the number of molecules of cysteine (or methionine) added to the reaction medium by DPM added to the same medium. The specific activity of 0.3 protein was determined by counting an aliquot of the purified protein and dividing DPM by the number of molecules in the aliquot ( $1 \text{ mg} = 5.4755 \times 10^{16}$  molecules). The molecules of cysteine (or methionine) incorporated per molecule 0.3 protein was calculated as shown in the legend to table 2.

Mistranslation of 0.3 protein - Results of cysteine misincorporation experiments are shown in Table 2. The range of values in 6 experiments using *E. coli* B was 0.021 to 0.024 cysteine residues misincorporated per molecule 0.3 protein synthesized and the mean  $\pm$  S.D. was  $0.023 \pm 0.001$ , or 23 cysteine residues misincorporated per 1000 molecules of 0.3 protein synthesized. Since there are 116 amino acids in 0.3 protein (14), one cysteine residue was misincorporated per  $5 \times 10^3$  codons translated in *E. coli* B. If all 20 amino acids were misincorporated at the same frequency as that of cysteine, there would be an average of one misincorporated amino acid per 250 codons translated or an error frequency of  $4 \times 10^{-3}$  per codon. This is a mistranslation level approximately 40-fold higher than was reported for the misincorporation of cysteine into *E. coli* flagellin (4). Evidence supporting our value for cysteine misincorporation into 0.3 protein comes from parallel methionine incorporation experiments. Using aliquots of the same *E. coli* and phage preparations and identical purification and calculation procedures we determined that 5.7

molecules of methionine were incorporated per molecule of 0.3 protein synthesized in T7 infected *E. coli* B (Table 2). The actual number is known from sequence data to be 6 (14).

$^{35}\text{S}$ -cysteine is not converted to  $^{35}\text{S}$ -methionine - Because the 0.3 protein contains methionine, metabolic conversion of  $^{35}\text{S}$ -cysteine to  $^{35}\text{S}$ -methionine would result in an erroneously high calculated error level for misincorporation of  $^{35}\text{S}$ -cysteine into 0.3 protein. It was therefore necessary to ensure that the 0.3 protein contained no  $^{35}\text{S}$ -methionine when  $^{35}\text{S}$ -cysteine was supplied as the precursor and that all the radioactivity in the 0.3 protein resided in cysteine residues. Thin layer chromatography was used to demonstrate directly that  $^{35}\text{S}$  remained exclusively in cysteine. The use of N-propanol: 34% ammonium hydroxide as solvent resolved cysteine and methionine into clearly separable spots which were located by ninhydrin staining (figure 3). Figure 3 shows that the  $^{35}\text{S}$ -label in  $^{35}\text{S}$ -cysteine-labeled 0.3 protein was contained only in cysteine and not in methionine residues.

Several experiments were undertaken using an *E. coli* strain (KL266) which, because of mutations, cannot interconvert cysteine and methionine. *E. coli* KL266 is defective in metE (tetrahydropteroyltriglutamate methyltransferase) and gysC (adenylsulfate kinase) and cannot form methionine from cysteine (24). Table 2 shows results of cysteine and methionine incorporation experiments using strain KL266. The value for methionine incorporation was similar to that obtained with strain B (5.0 vs. 5.7 molecules/molecule 0.3 protein). The value for cysteine misincorporation was, however, slightly lower than that obtained with strain B (0.014 vs. 0.023 molecules cysteine incorporated per molecule 0.3 protein synthesized). Note that *E. coli* KL266 carries a mutation in ribosomal protein S12 (rpsL). Mutations in this protein have been shown to increase the fidelity of translation (11, 25) which may account for the slight decrease in

cysteine misincorporation in T7-infected KL266 cells.

Aminoglycoside-Induced Misreading - The aminoglycoside antibiotics, streptomycin and gentamicin, are known to promote mistakes in translation. It would be expected that higher levels of  $^{35}\text{S}$ -cysteine misincorporation into 0.3 protein would be observed if either of these antibiotics were present during labeling of T7-infected cells. Experiments performed with T7-infected *E. coli* B demonstrated that increasing amounts of gentamicin caused increasing amounts of cysteine <sup>To be</sup> misincorporated (table 3). When 2.5  $\mu\text{g}$ , 5.0  $\mu\text{g}$ , and 10  $\mu\text{g}$  gentamicin sulfate were added per ml, cysteine misincorporation was 126%, 152% and 165%, respectively, of that incorporated in the absence of gentamicin.

These results were confirmed in  $^{35}\text{S}$ -cysteine misincorporation-RIP experiments. SDS-PAGE and autoradiographic analyses of the immune precipitates showed increasing  $^{35}\text{S}$ -cysteine incorporation into 0.3 protein with increasing amounts (10 to 40  $\mu\text{g}/\text{ml}$ ) of gentamicin sulfate present (figure 4). Figure 4 clearly shows that whereas overall protein synthesis decreased with increasing gentamicin concentration, radioactivity (present in  $^{35}\text{S}$ -cysteine) in the 0.3 protein increased.

We performed similar cysteine misincorporation experiments using streptomycin. Strains of *E. coli* were constructed which were isogenic except for the presence or absence of specific mutations known to affect ribosomal proteins which play a role in the fidelity of protein synthesis. RL002 has wild type ribosomes, RL003 carries a mutation in *rpsE* which decreases fidelity in translation (ribosomal ambiguity mutant = ram), DS410 carries a mutation of *rpsL* which increases fidelity of translation in the presence of streptomycin. Results are shown in table 3. When strain RL002 served as host, addition of streptomycin sulfate (10  $\mu\text{g}/\text{ml}$ ) resulted in cysteine misincorporation which was 144% of that seen in identical experiments without streptomycin. As expected,

when strain DS410 served as host, there was no difference in mistranslation frequency in the presence or absence of streptomycin. When strain RL003 (ram) served as host, in the absence of streptomycin cysteine misincorporation was 136% of that observed with strain RL002 (wild-type). In the presence of streptomycin, even more cysteine was misincorporated into 0.3 protein - consistent with the known streptomycin hypersensitivity of the ramC319 mutation (25).

## DISCUSSION

The T7 0.3 protein appears to be well suited for studies of in vivo mistranslation. It is synthesized in large amounts and only after T7 infection, is relatively easy to purify, contains no cysteine, and its precise amino acid sequence and codon usage are known (13, 14). Using the T7 0.3 protein, we have found the level for misincorporation of cysteine into this protein to be 0.023 residues per molecule. If other amino acids are misincorporated at a similar rate, the overall mistranslation level for 0.3 protein in T7-infected *E. coli* cells would be  $4 \times 10^{-3}$  misincorporations per codon translated. This is 40 times higher than the error rate determined by Edelman and Gallant (4) for cysteine misincorporation into *E. coli* flagellin. It is similar, however, to the level found by Parker et al. (15) for misincorporation of lysine for asparagine in MS2 phage coat protein ( $2 \times 10^{-3}$ ) and to the level found by Rosenberger et al. (7) for the misreading of a nonsense codon in *E. coli* alkaline phosphatase ( $2 \times 10^{-3}$ ). It is satisfying that a similar (albeit high) error level was found for 3 different proteins by 3 different techniques.

Theoretically, errors in proteins synthesized in vivo could occur at the levels of DNA replication (mutation) and transcription, as well as translation. Mutation is not a likely source of the error we are measuring; mutation rates are  $10^5$ - $10^7$  lower than the rate of cysteine misincorporation into 0.3 protein (26). Mistakes in transcription, on the other hand, probably do contribute to the overall error rate in any experimental system which measures in vivo mistranslation. However, the contribution of mistranscription to experimentally determined errors in translation is probably minor because mistranscription levels have been observed to be at least 10 fold lower than mistranslation levels (26). That we are predominantly measuring translational errors was shown by: 1) experiments in which addition of aminoglycoside antibiotics known to induce misreading resulted in increased amounts of cysteine misincorporated into



0.3 protein (table 3 and figure 4) and 2) by experiments utilizing constructed strains of *E. coli* isogenic except for mutations in ribosomal protein genes. In each case, the error level was increased or decreased as predicted by the known effect of the mutation on the fidelity of translation (table 3).

The frequency of translational errors in vivo was found by other workers to be increased by 4 to 50 fold by the presence of aminoglycoside antibiotics (4,7,27,28). The absolute error level observed was similar to the level we found for misincorporation of cysteine into 0.3 protein in the presence of gentamicin or streptomycin ( $5-7 \times 10^{-3}$ /codon, depending upon antibiotic concentration (table 3)). In our experiments the cysteine misincorporation level without antibiotics was  $4 \times 10^{-3}$ /codon, making the increase in errors caused by addition of antibiotics modest (less than two-fold) even at the highest concentration of antibiotics used (10  $\mu$ g/ml). This suggests that the decrease in fidelity caused by aminoglycosides is relative and may depend upon the natural error level of a particular amino acid misinsertion, with the antibiotic exerting a lesser effect in the case of a normally high error level. The highest concentration of gentamicin and streptomycin used in these experiments (10  $\mu$ g/ml) still allowed sufficient synthesis of 0.3 protein for purification and accurate quantitation of  $^{35}$ S-cysteine misincorporation. At gentamicin concentrations greater than 10  $\mu$ g/ml we may have found higher levels of cysteine misincorporation as suggested by the RIP-PAGE experiment shown in figure 4.

We do not know if other amino acids are misincorporated into 0.3 protein at the same rate as is cysteine. There is evidence to suggest that there is a hierarchy of errors which can occur on the ribosome (10,11,26,29,30). The probability of mistranslation of a particular codon will depend not only on that codon (which will have codon-anticodon interactions of varying strengths with cognate and noncognate tRNAs (31-34), but also on its neighboring codons within

the mRNA being translated (35). The probability of substitution varies from site to site within a protein and thus different proteins, as a whole, may have different error rates. The fact that experimentally determined frequencies of *in vivo* mistranslation differ by more than ten-fold must reflect at least some of these variables. A factor which may also influence experimental results is the stability of the error-containing protein being studied. It is known that half-lives of polypeptides with abnormal structures are, in general, much shorter than the half-lives of most correctly synthesized polypeptides in bacterial and eukaryotic cells (36-37). We have used the 0.3 protein of T7 phage to assay mistranslation; therefore the relatively high error frequency we observed may reflect the fact that degradation of abnormal proteins is known to be inhibited in cells infected with T7 phage (38).

A major unresolved question is whether cysteine substitutes only for arginine due to first position codon misreading as suggested by Edelman and Gallant (4), or whether other types of errors (2nd or 3rd position misreading) can also occur. There are 4 arginine residues in 0.3 protein and they fall into only two cyanogen bromide (CNBr) cleavage products. We are currently determining which CNBr fragments of 0.3 protein contain <sup>35</sup>S-cysteine. The 0.3 gene has been cloned on a plasmid which replicates in *E. coli* (\*) and on a phage which replicates in *B. subtilis* (\*) so it will also be possible to determine whether the frequency of cysteine misincorporation into 0.3 protein is different in non-infected *E. coli* and in another procaryotic species, *B. subtilis*.

### Figure Legends

Figure 1. Methionine and cysteine-containing polypeptides of T7<sup>+</sup> (wild type) bacteriophage. Minicells isolated from *E. coli* RL002 ( $2 \times 10^9$ ) were infected with T7<sup>+</sup> phage at an input m.o.i. of 5 and incubated for 30 min at 37°C in the presence of <sup>35</sup>S-methionine or <sup>35</sup>S-cysteine. Radioactively-labeled polypeptides were detected by fluorography after preparation for and separation by SDS-PAGE as previously described (19, 20). Track a=<sup>35</sup>S-methionine labeled T7 polypeptides; track b=<sup>35</sup>S-cysteine labeled T7 polypeptides. T7 late polypeptides 9 and 16 and early polypeptide 0.3 do not contain cysteine.

Figure 2. SDS-Polyacrylamide gel electrophoresis of purified 0.3 protein. T7 0.3 protein (from 3 experiments) was purified by DEAE cellulose chromatography and ethanol extraction and examined for purity by SDS-PAGE and the silver nitrate stain as described in Experimental Procedures. In the 3 leftmost tracks were applied 2 µg of the proteins recovered from the ethanol supernatants; in the 3 rightmost tracks were applied aliquots of the proteins recovered from the ethanol precipitates. Every other lane of the gel contained sample buffer only. The dark bands near the top of the gel are an artifact of sample buffer and the silver stain technique.

Figure 3. Autoradiogram of thin layer chromatography of hydrolysed <sup>35</sup>S-cysteine-labeled 0.3 protein. Purified 0.3 protein, synthesized in the presence of <sup>35</sup>S-cysteine was hydrolyzed in 6N HCl. The dried hydrolysate was resuspended in water and unlabeled cysteine-HCl (12 µg) and methionine (15 µg) were added as markers. The mixture (7 µl) was spotted on a cellulose sheet and developed with N-propanol: 34% NH<sub>4</sub>OH (7:3) (left track). Spotted in the right track was 1 µl of a dilution of <sup>35</sup>S-methionine equal to 7000 DPM plus 15 µg

unlabeled methionine. Following chromatography the chromatogram sheet was used to expose X-ray film (X-OMAT, Kodak Company) to locate  $^{35}\text{S}$ -cysteine and  $^{35}\text{S}$ -methionine. The locations of these radioactive spots were compared to the ninhydrin-determined locations of cysteine (dotted spot, left track) and methionine (dotted spot, right track).

Figure 4. Increased misincorporation of  $^{35}\text{S}$ -cysteine into 0.3 protein with increased gentamicin concentration. *E. coli* B was infected with T7  $\Delta$  H3, 1am193 $\Delta$  LG3 in the presence or absence of gentamicin sulfate. The cell lysates were reacted with either non-immune serum (tracks 1a, 2a, 3a and 4a) or anti-0.3 antiserum (tracks 1b, 2b, 3b and 4b) and the precipitated material analyzed by fluorography following SDS-PAGE as described in Experimental Procedures. Tracks 1a and 1b = no gentamicin present; tracks 2a and 2b = 10  $\mu\text{g}$  gentamicin/ml; tracks 3a and 3b = 20  $\mu\text{g}$  gentamicin/ml; tracks 4a and 4b = 40  $\mu\text{g}$  gentamicin/ml.

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Footnotes

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Table 1. Strains of *E. coli* used in this study.

<u>Strain</u>	<u>Genotype</u>	<u>Source</u>
B	Su <sup>0</sup>	Lab stock
BBw/t	Su <sup>+</sup> (amber suppressor of unknown specificity)	Lab stock
KL266	<u>leu B6</u> , <u>proC32</u> , <u>hisF80</u> , <u>cysC43</u> , <u>thyA54</u> , <u>metE70</u> , <u>thi-1</u> , <u>ara-14</u> , <u>lacZ36</u> , <u>xyl-5</u> , <u>mtl-1</u> , <u>malA38</u> , <u>rpsE2115</u> , <u>rpsL109</u>	K.B. Low via B.J. Bachmann
RL001	<u>minA</u> , <u>minB</u> , <u>aroE</u> , (spc <sup>r</sup> )	Constructed for this work <sup>a</sup>
RL002	<u>minA</u> , <u>minB</u>	Constructed for this work <sup>b</sup>
RL003	<u>minA</u> , <u>minB</u> , <u>ramC319</u> , (spc <sup>s</sup> )	Constructed for this work <sup>c</sup>
DS410	<u>minA</u> , <u>minB</u> , <u>rpsL</u>	J.N. Reeve (20)
C600	<u>supE</u>	
RH2887	<u>thr</u> <sup>-</sup> , <u>leu</u> <sup>-</sup> , <u>lacY</u> , <u>ramC319</u> , <u>supE</u>	T. Cabezón (25)
AB2834	<u>aroE</u> , (spc <sup>r</sup> )	J. Davies

<sup>a</sup> Plvir transduction of aroE, spc<sup>r</sup> from *E. coli* AB2834 to DS410. Spc<sup>r</sup> transductants were selected and screened for aroE and rpsL<sup>+</sup>. This strain was used to construct RL 003.

<sup>b</sup> Plvir transduction of rpsL<sup>+</sup> from *E. coli* C600 to DS410. Transductants were replica plated from M9 minimal medium onto M9 minimal medium plus streptomycin (10 ug/ml) to isolate RL002, a streptomycin-sensitive, minicell-producing strain.

<sup>c</sup> Plvir transduction of *E. coli* RH2887 into RL001, selecting aroE<sup>+</sup> transductants on minimal agar plates lacking the aromatic amino acids. AroE<sup>+</sup> transductants were subsequently screened on minimal agar plates containing spectinomycin (100 ug/ml). AroE<sup>+</sup>, spc<sup>s</sup> transductants were analyzed for the ramC319 allele by their ability to suppress both T4 nonsense mutants HB7 (UAG codon) and N65 (UGA codon) (25).

Table 2. Incorporation of Cysteine and Methionine into T7 0.3 Protein.

<u>Strain</u>	<u>Molecules of cysteine per molecule 0.3 protein<sup>a</sup></u>	<u>Molecules of methionine per molecule 0.3 protein</u>
B	$0.023 \pm 0.001^b$	$5.7 \pm 0.6^d$
KL266	$0.014 \pm 0.002^c$	$5.0 \pm 1.7^d$

<sup>a</sup> Experiments were performed as described in Experimental Procedures. Molecules of cysteine (or methionine) per molecule 0.3 protein was calculated as: DPM in 1 molecule of 0.3 protein X Number of cysteine (or methionine) molecules per <sup>35</sup>S DPM.

<sup>b</sup> Mean of 6 experiments  $\pm$  S.D.

<sup>c</sup> Mean of 4 experiments  $\pm$  S.D.

<sup>d</sup> Mean of 3 experiments  $\pm$  S.D.

Table 3. Increased Misincorporation of Cysteine into 0.3 Protein in the Presence of Gentamicin or Streptomycin.

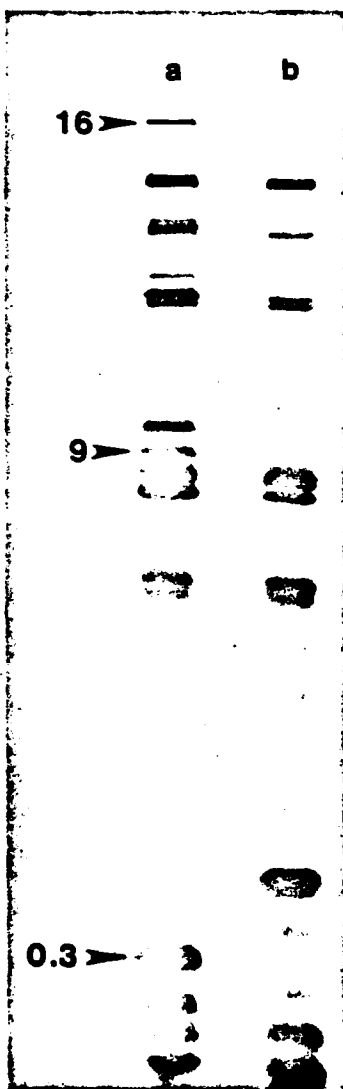
<u>Strain</u>	<u>µg gentamicin sulfate/ml<sup>a</sup></u>	<u>Molecules of cysteine per molecule 0.3 protein<sup>b</sup></u>
B	0	0.023
B	2.5	0.029
B	5.0	0.035
B	10.0	0.038

<u>Strain</u>	<u>Phenotype<sup>c</sup></u>	<u>µg streptomycin/ml</u>	<u>Molecules of cysteine per molecule 0.3 protein<sup>b</sup></u>
RL002	W.T.	0	0.025
RL002	W.T.	10	0.036
DS410	str <sup>r</sup>	0	0.026
DS410	str <sup>r</sup>	10	0.028
RL003	ram	0	0.034
RL003	ram	10	0.041

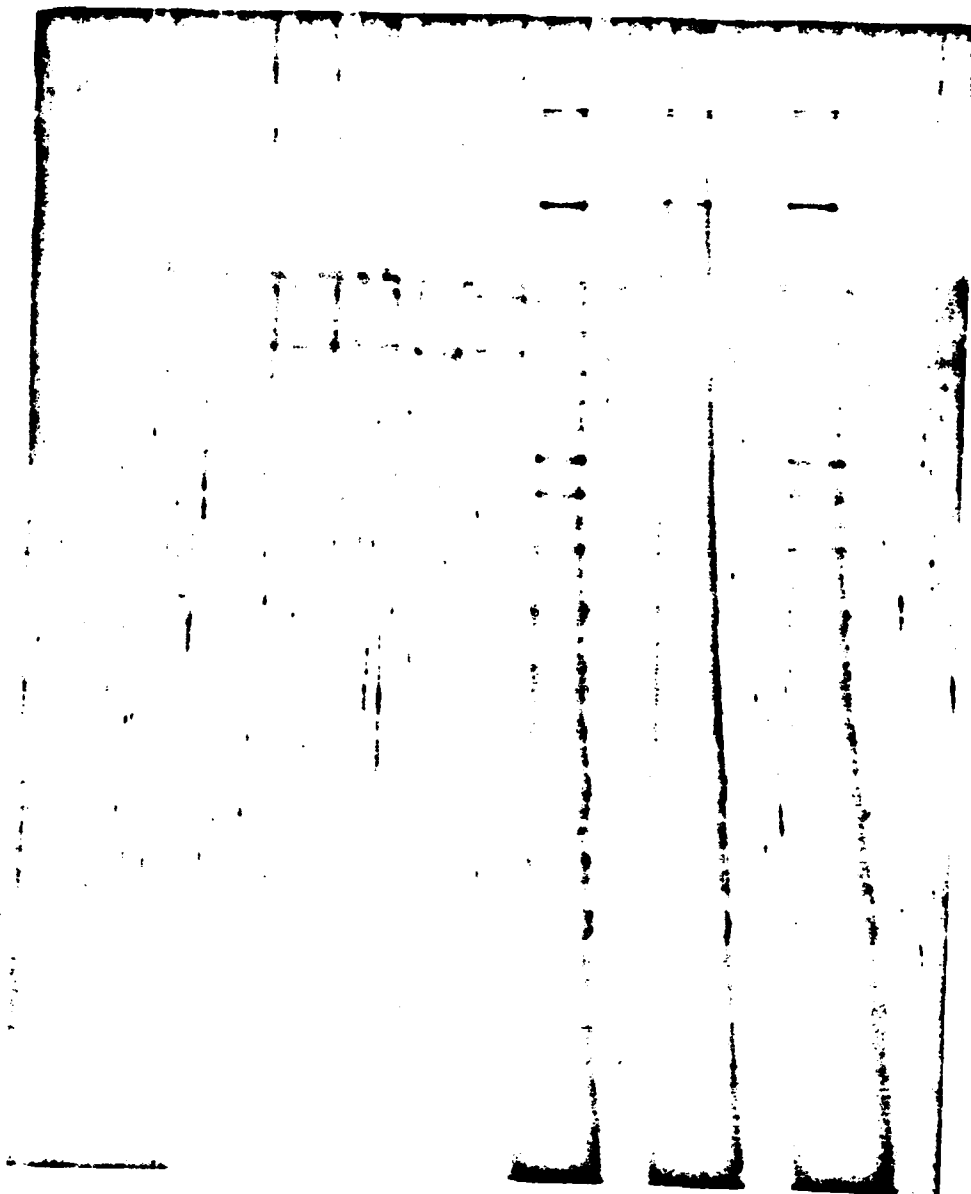
<sup>a</sup>Potency (ug/ug) as determined by the manufacturer was 61%.

<sup>b</sup>Calculations were performed as described in Table 2.

<sup>c</sup>Phenotype for protein synthesis: W.T. = wild type; str<sup>r</sup> = streptomycin-resistant; ram = ribosomal ambiguity mutant.

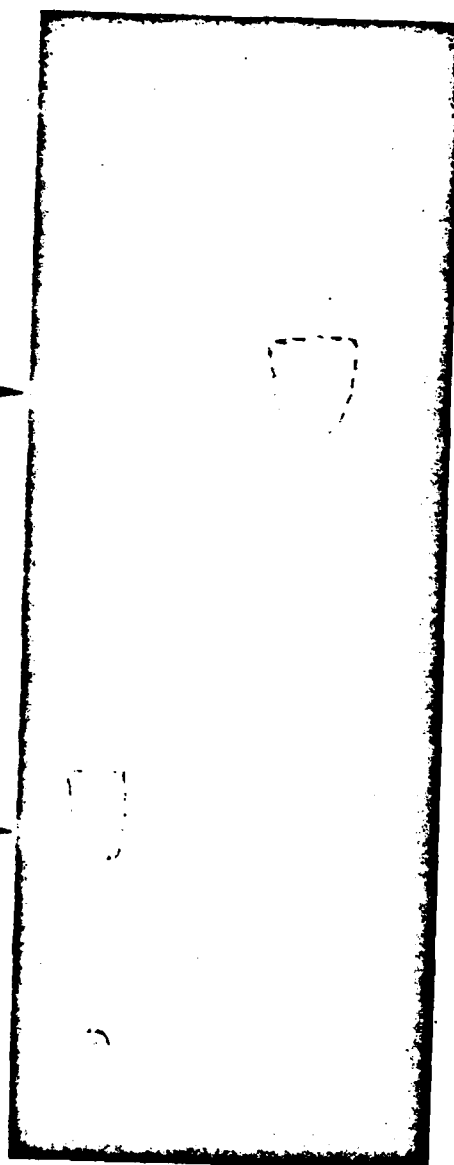


0.3

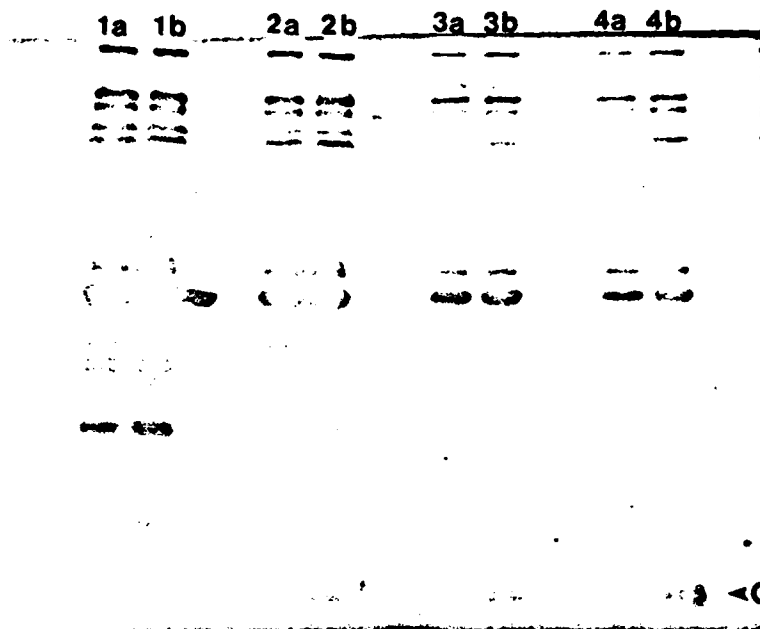


MET➤

CYS➤



6



L. Biographical Sketch

NAME: John N. Reeve  
Professor

SOCIAL SECURITY NO.  
527-19-4213

DATE AND PLACE OF BIRTH: Wakefield, England; 6/21/47

ALIEN REGISTRATION NUMBER: A35395844 (Permanent Resident)

Married; Two children (Born 4/74; 12/78)

EDUCATION:

University of Birmingham, England, B.Sc., 1968, Bacteriology  
University of British Columbia, Canada, Ph.D., 1971, Microbiology

THESIS TITLE: Cell division in a temperature-sensitive mutant of *E. coli*.

HONORS:

Commonwealth Universities Scholarship  
NATO postdoctoral Fellowship  
EMBO postdoctoral Fellowship  
N.I.H. Research Career Development Award (12/80-11/85)  
Distinguished Visiting Research Professorship, University of Adelaide  
Adelaide, Australia (1982).

MAJOR RESEARCH INTEREST:

Microbial Physiology/Regulation of Macromolecular Syntheses/Microbial  
Genetics; Bacterial Virus Development; Genetics of Methane Producing  
Micro-organisms; Aging

RESEARCH EXPERIENCE:

1981 - Professor, Department of Microbiology, The Ohio State University  
1979-1981 - Associate Professor, Department of Microbiology, The Ohio  
State University  
1974-1979 - Research Group Director, Max Planck Institut fur Molekulare  
Genetik, 1 Berlin 33, Germany. Direction of graduate students,  
technicians in research projects. Provision of courses in microbial  
genetics and physiology. Reviewing manuscripts submitted as  
research papers or grant proposals. Responsible for control of  
recombinant DNA research.  
1979 - Instructor in EMBO advanced techniques course (Plasmids).  
Erlangen, W. Germany.  
1973 - Postdoctoral fellow with Dr. Howard J. Rogers, Department of  
Microbiology, National Institute for Medical Research, Mill  
Hill, London, England. Research in bacterial cell wall genetics  
and biochemistry.  
1971-1973 - Postdoctoral fellow with Dr. Neil H. Mandelsohn, Department  
of Microbiology, University of Arizona, Tucson. Research in bac-  
terial cell growth. Isolation and characterization of mutants in-  
cluding the minicell mutants of *E. subtilis*.

PROFESSIONAL SERVICE:

Grant reviews (N.I.H., N.S.F., N.A.T.O., D.O.E.)  
Project consultant (D.O.E.)  
Manuscript reviews (J. Mol. Biol.; J. Virol.; Mol. Gen. Genet.;  
Curr. Microbiol.; Eur. J. Biochem.; J. Bacteriol.)



# PUBLICATIONS:

1. Reeve, J.N., D.J. Groves and D.J. Clark. 1970. Regulation of cell division of Escherichia coli. Characterization of temperature-sensitive division mutants. J. Bacteriol. 104:1052-1064.
2. Reeve, J.N., R.A. Weigand and J.W. Grenawalt. 1971. Presence of bacteriophage-like inhibitory particles in Escherichia coli. J. Bacteriol. 107:926-928.
3. Reeve, J.N. and D.J. Clark. 1972. Cell division of Escherichia coli HUG-6: Effect of varying the growth period at the non-permissive temperature. J. Bacteriol. 110:117-121.
4. Reeve, J.N. and D.J. Clark. 1972. Cell division of Escherichia coli HUG-6: Effect of varying the temperature used as the nonpermissive growth condition. J. Bacteriol. 110:122-125.
5. Reeve, J.N., N.H. Mendelson and R.M. Cole. 1972. Cell morphology of Bacillus subtilis: The effect of genetic background on the expression of a rod gene. Molec. Gen. Genet. 119:11-26.
6. Reeve, J.N., N.H. Mendelson, S.I. Coyne, L.L. Hallock and R.M. Cole. 1973. Minicells of Bacillus subtilis. J. Bacteriol. 114:860-873.
7. Mendelson, N.H. and J.N. Reeve. 1973. Growth of the Bacillus subtilis cell surface. Nature (London), 243:62-64.
8. Reeve, J.N. and N.H. Mendelson. 1973. Minicells of Bacillus subtilis. A new phage blocking agent. J. Virol. 12:944-945.
9. Reeve, J.N. and N.H. Mendelson. 1973. Pronase digestion of amino acid binding components on the surface of Bacillus subtilis cells and minicells. Biochem. Biophys. Res. Comm. 53:1325-1330.
10. Reeve, J.N. and N.H. Mendelson. 1974. Minicells of Bacillus subtilis: A unique system for transport studies. Biochem. Biophys. Acta 352:298-307.
11. Mendelson, N.H., J.N. Reeve and R.M. Cole. 1974. Physiological studies of Bacillus subtilis minicells. J. Bacteriol. 117:1312-1319.
12. Reeve, J.N. 1974. Growth of the Bacillus subtilis cell surface. J. Bacteriol. 119:560-563.
13. Rogers, H.J., P.F. Thurman, C. Taylor and J.N. Reeve. 1974. Mucopolysaccharide synthesis by rod mutants of Bacillus subtilis. J. Gen. Microbiol. 81:335-350.
14. Reeve, J.N. and J.B. Cornett. 1975. Bacteriophage SP01-induced macromolecular synthesis in minicells of Bacillus subtilis. J. Virol. 15:1308-1316.
15. Reeve, J.N. 1976. Macromolecular synthesis in bacteriophage infected minicells of Bacillus subtilis. Microbiology 1976, ASM Publications p. 332-339.

16. Martens, G. and J.N. Reeve. 1977. Synthesis of cell envelope components by anucleate cells (minicells) of Bacillus subtilis. J. Bacteriol. 129:1198-1207.
17. Reeve, J.N. 1977. Mucopolysaccharide biosynthesis by minicells of Escherichia coli. J. Bacteriol. 131:363-365.
18. Ponta, H., J.N. Reeve, J. Pfenning, M. Hirsch-Kaufmann, M. Schweiger and P. Herrlich. 1977. T7 development in F+ E. coli cells and anucleate minicells. Nature. 269:440-442.
19. Reeve, J.N. 1977. A general method for the "in vivo" analysis of bacteriophage encoded products. Molec. Gen. Genetics 158:73-79.
20. Reeve, J.N., G. Martens and E. Amann. 1978. Early development of bacteriophages SP01 and SP82G in minicells of Bacillus subtilis. J. Mol. Biol. 120:183-207.
21. Amann, E. and J.N. Reeve. 1978. DNA synthesis in bacteriophage infected toluene treated minicells of B. subtilis. Biochim. Biophys. Acta 520:82-87.
22. Magazin, M., J.N. Reeve, S. Maynard-Smith and N. Symonds. 1978. Bacteriophage M1 encoded polypeptides synthesized in infected minicells. FEMS Lett. 4:5-9.
23. Subramanian, A.R. and J.N. Reeve. 1978. Synthesis of ribosomal proteins in drift 18 infected minicells of Escherichia coli and selective incorporation into ribosomes. FEMS Lett. 25:265-269.
24. Reeve, J.N. 1978. Selective expression of transduced or cloned DNA in minicells containing plasmid pKB280. Nature. 276:728-729.
25. Martens, G., E. Amann and J.N. Reeve. 1979. Bacteriophage SP01 polypeptides synthesized in infected minicells and in vitro. Molec. Gen. Genet. 172:271-279.
26. Reeve, J.N. and J.E. Shaw. 1979. Lambda encodes an outer membrane protein: the lom gene. Molec. Gen. Genet. 172:243-248.
27. Reeve, J.N., Lanka, E. and Schuster, H. 1980. Synthesis of P1han protein in minicells infected by P1 mutants. Molec. Gen. Genet. 177:193-197.
28. Reeve, J.N., Amann, E.P., Toller, R., Gunthert, U., Scholz, K. and Trautner, T.A. 1980. Unusual behaviour of SP01 DNA with respect to restriction and modification enzymes recognizing the sequence 5'-G-G-C-C-. Molec. Gen. Genet. 178:229-231.
29. Reilmann, H., Reeve, J.N. and Puhler, A. 1980. Identification of the repressor and repressor bypass (antirepressor) polypeptides of bacteriophage P1 synthesized in infected minicells. Molec. Gen. Genet. 178:149-154.

30. Heilmann, H., Puhler, A., Burkhardt, H.-J. and Reeve, J.N. 1980. Transposon mutagenesis of the gene encoding the bacteriophage P1 restriction endonuclease. Colinearity of the gene and gene product. *J. Mol. Biol.* **144**:387-396.
31. Nothling, R. and Reeve, J.N. 1980. Protein synthesis results in guanosine-5'-diphosphate-3'-diphosphate (ppGpp) synthesis in minicells of *Escherichia coli*. *J. Bacteriol.* **143**:1060-1062.
32. Giphart-Gassler, M., Reeve, J.N. and P. van de Putte. 1981. Polypeptides encoded by the early region of bacteriophage Mu synthesized in minicells of *Escherichia coli*. *J. Mol. Biol.* **145**:165-191.
33. Giphart-Gassler, M., Wijffelman, C. and Reeve, J.N. 1981. Structural polypeptides and products of late genes of bacteriophage Mu Characterization and functional aspects. *J. Mol. Biol.* **145**:139-163.
34. Amann, E.P., J.N. Reeve, G. Morelli, B. Behrens and T.A. Trautner. 1981. Cloning and expression of the *Bacillus subtilis* phage SPPI in *E. coli*. I. Construction and characterization of /SPPI hybrids. *Molec. Gen. Genet.* **182**:293-298.
35. Amann, E.P. and J.N. Reeve. 1981. Cloning and expression of the *Bacillus subtilis* phage SPPI *E. coli*. II. Expression of /SPPI hybrids phages in *E. coli* minicells. *Molec. Gen. Genet.* **182**:299-303.
36. Reeve, J.N. 1981. OX174 directed DNA and protein synthesis in mini cells. *J. Virology.* **40**:396-402.
37. Brunel, F., J. Davison, V. Ha Thi and J.N. Reeve. 1981. Cloning of bacteriophage T5 DNA fragments. Expression of cloned T5 DNA fragments in *Escherichia coli* minicells. *Gene.* **16**:371-383.
38. Heilmann, H. and J.N. Reeve. 1982. Construction and use of SPPIV, a cloning vector for *Bacillus subtilis*. *Gene* **17**:91-100.
39. Hamilton, P.T. and J.N. Reeve. 1983. Cloning and expression of Archaeobacterial DNA from methanogens in *Escherichia coli*. In "Microbial Chemosutotrophy"; ed. Strohl, W.R. and Tuovinen, O.R., Ohio State Press, Columbus, Ohio. In Press.
40. Hook, L.E., R.E. Corder, P.T. Hamilton, J.I. Frea, and J.N. Reeve. 1983. Development and use of an ultra-low oxygen chamber for the growth of methanogens. In "Microbial Chemosutotrophy", ed. W.R. Strohl and O.R. Tuovinen, Ohio State Press, Columbus, Ohio. In Press.
41. Shaw, J.E., Epp, C., Reeve, J.N. and Pearson, M.L. 1982. Differences in the regulation of phage lambda protein synthesis and degradation in UV-irradiated *E. coli* and in minicells. *Virology*. Submitted for Publication.
42. Hamilton, P.T. and Reeve, J.N. 1982. Cloning and expression of archaeobacterial DNA from methanogens in *Escherichia coli*. *Nature*. Submitted for Publication.

43. Puspurs, A.H., N.J. Trun, and J.N. Reeve. 1983. Bacteriophage Mu DNA circularizes following infection of Escherichia coli E.M.B.O. Journal, In Press.

#### REVIEW ARTICLES

1. Reeve, J.N. 1979. Use of minicells for bacteriophage-directed polypeptide synthesis. *Methods in Enzymol.* n68n:493-511.
2. Reeve, J.N. 1979. Isolation of minicells and characterization of plasmid and phage encoded polypeptides by SDS-polyacrylamide gel electrophoresis. EMBO Advanced Techniques Course on Plasmids. Erlangen, W. Germany. pp. 130-151.
3. Reeve, J.N., N. Trun, and P. Hamilton. 1981. Beginning genetics of methanogens. In 'Genetic Engineering of Micro-organisms for Chemicals.' Ed. A. Hollaender. Assoc. Univ. Press., Wash., DC 20036. pp.233-244.

#### NON-REVIEWED ARTICLES AND ABSTRACTS OF MEETINGS

These are not included in the attached list of publications as I do not consider them to be publications. The details could be supplied (approximately 50 citations) if needed.

CURRICULUM VITAE

October , 1983

NAME: Jacqueline Bowers Rice

BORN: June 10, 1938

MARITAL STATUS: Married

EDUCATION:

B.A. University of Wisconsin-Milwaukee, 1960.

Registered Medical Technologist, ASCP, 1961  
Milwaukee Lutheran Hospital Internship, 1960-1961.

Graduate Program, Department of Medical Microbiology,  
1967-1968, Marquette University, Milwaukee, Wisconsin.

Graduate Program in Molecular Biology, Department of  
Biology, Marquette University, Milwaukee, Wisconsin,  
1968-1970.

Graduate Program, Department of Medical Microbiology,  
Ohio State University, Columbus, Ohio, 1972-1977.

Doctor of Philosophy: Research was directed by Dr.  
Raymond Lang whose work has involved several transplanta-  
tion models.

Dissertation Title: Extraction of xenotransplantation  
antigens and their use in xenograft prolongation and  
studies of xenograft rejection.

TEACHING EXPERIENCE:

Medical Student Independent Study Program, Ohio State  
University, 1975. (Conducted laboratory exercises in  
medical microbiology and infectious diseases.)

Laboratory Instructor for the medical student Medical  
Microbiology course (MM 625), Ohio State University, 1973.

Teaching Assistant (Genetics), Department of Biology,  
Marquette University, 1969.

APPOINTMENTS:

Research Associate II, Ohio State University, Department of Microbiology, College of Biological Sciences, 1981-present.

Research Associate I, Ohio State University, Department of Veterinary Pathobiology, College of Veterinary Medicine, 1977-1981.

Graduate Research Associate, Ohio State University, Department of Medical Microbiology, 1973-1975.

Medical Technologist, Mount Carmel Hospital, Columbus, Ohio, 1971.

Medical Technologist, Deaconess Hospital, Milwaukee, Wisconsin, 1970.

NIH Traineeship in Developmental Biology, 1968-1970.

NDEA Title IV Fellowship (NIH), 1967-1968.

Medical Technologist, Milwaukee County Hospital, Milwaukee, Wisconsin, 1964-1967.

Medical Technologist, San Diego County Hospital, San Diego, California, 1961-1963.

HONORS AND AWARDS:

Science Honorary Fraternity (Delta Chi Sigma), University of Wisconsin-Milwaukee, 1958-1960.

Graduated With Honors, University of Wisconsin-Milwaukee, 1960.

NDEA Title IV Fellowship (NIH), Marquette University, 1967-1968.

NIH Traineeship in Developmental Biology, 1968-1970.

GRANTS AWARDED:

USPHS, NIH, I-R01-CA30338-01, FeLV leukemogenesis and pre-neoplastic lesions, co-investigator, 3 yrs., \$180,895 total, 11/81-10/84. (I wrote the entire grant proposal. Richard G. Olsen is listed as P.I. because of University regulations.)

The Ohio State Canine Research Funds, "Immunoprevention of Parvovirus-Induced Diarrhea of the Dog", 2 yrs., \$20,000/yr., 1981-1982. (I wrote the entire grant proposal. Richard G. Olsen and G. Stephen Krakowka are listed as P.I.'s because of University regulations.)

PUBLICATIONS:

Rice, J.B.: Extraction of xenotransplantation antigens and their use in xenograft prolongation and studies of xenograft rejection. Ph.D. dissertation, 1977.

Rice, J.B. and Lang, R.W.: Solubilization and partial characterization of rat lymphocyte antigen. American Society for Microbiology, abstract, 1976.

Rice, J.B., Schaller, J.P., and Olsen, R.G.: FOCMA expression in preneoplastic FeLV-induced lesions. American Society for Cancer Research, abstract, 1980.

Olsen, R.G., Rice, J.B., and Stiff-Torvik, M.: Feline leukemia precancerous lesion, in Feline Leukemia Virus, Hardy, W.D., Jr., Essex, M., and McClelland, eds., Elsevier, North Holland, 1980, pp. 133-140.

Rice, J.B., Schaller, J.P., Lewis, M.B., Mathes, L.E., Hoover, E.A., and Olsen, R.G.: Infection of feline embryo adherent cells with FeLV: FOCMA expression and morphologic transformation, J. Natl. Cancer Inst., 66:89-96, 1981.

Rice, J.B. and Olsen, R.G.: FeLV/FeSV-associated transformation specific protein(s), in Feline Leukemia, R.G. Olsen, ed., CRC Press, Boca Raton, Florida, 1981, pp. 53-67.

Rice, J.B. and Olsen, R.G.: Early expression of feline oncornavirus-associated cell membrane antigen and persistent gsa expression following feline leukemia virus infection, in Advances in Comparative Leukemia Research, D.S. Yohn and J.R. Blakeslee, eds., Elsevier Science Publishing Co., The Netherlands, 1981, pp. 223-224.

Rice, J.B. and Olsen, R.G.: Feline oncornavirus-associated cell membrane antigen (FOCMA) and FeLV gsa expression in bone marrow and serum. J. Natl. Cancer Inst., 66:737-743, 1981.

Krakovka, S., Olsen, R.G., Axthelm, M.K., Rice, J.B., and Winters, K.A.: Canine parvovirus infection potentiates canine distemper encephalitis attributable to modified live virus vaccine. J. Am. Vet. Med. Assoc., 180:137-139, 1982.

Rice, J.B., Krakowka, S., Winters, K.A., and Olsen, R.G.: Comparison of systematic and local immunity in dogs with canine parvovirus gastroenteritis, J. Inf. Immun., 38: 1003-1009, 1982.

Rice, J.B. and Reeve, J.N.: Mistranslation of a T7 Bacteriophage protein, submitted for publication (10/83).

DATE  
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